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The Journal of LABORATORY and CLINICAL MEDICINE

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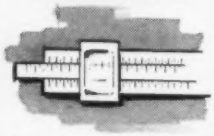

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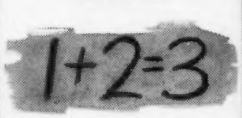

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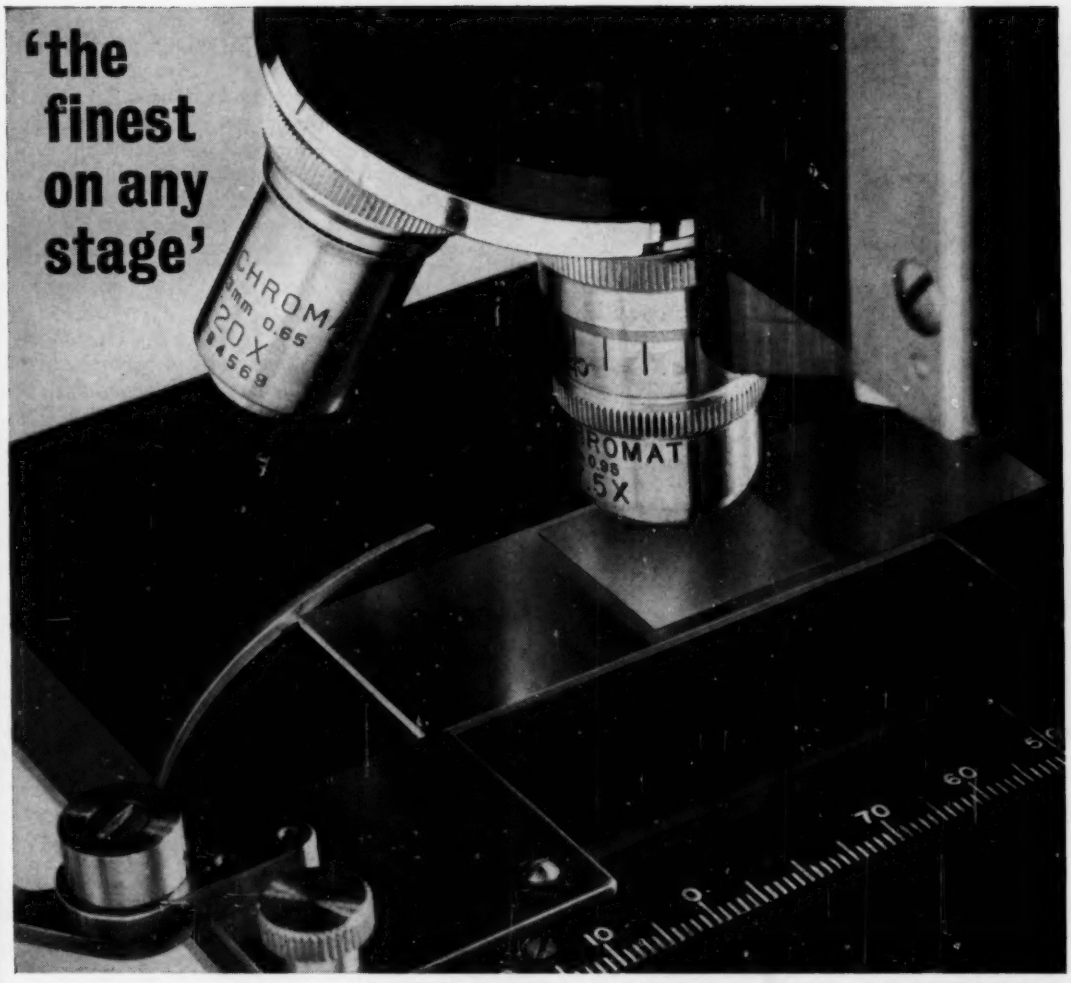

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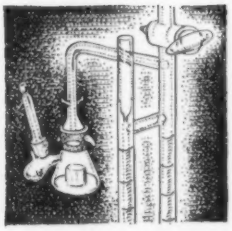

IN JULY—(1873)—Joseph Lister reviews a series of experiments supporting the germ theory of fermentation. This work utterly disproves the rival oxygen theory and shows all reported instances of spontaneous generation have been due to faulty experimentation. There is no evidence that any chemical possesses the one characteristic that distinguishes all true fermentation—self-propagation. However, it has been shown that emulsin, a peculiar albuminous principle existing in sweet and bitter almonds, can break up ten times its weight of amygdalin. "In this sense, then, as intervening between the growth of organisms and the resultant decompositions, the theory of chemical ferments might be welcomed as a valuable hypothesis."[1]

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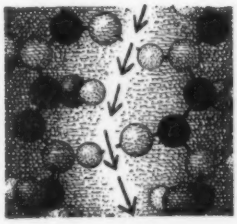

IN JULY—(1933)—a report from the Lister Institute (London) considers the effect of proteolytic enzymes on the oxytocic hormone. Gulland and Macrae[2] find that two commercial trypsin preparations inactivate this hormone at widely different rates. A comparison experiment using quantities with equal tryptic activity clearly indicates that the inactivating enzyme is not trypsin. Nor is it a papainase, arginase, or prolinase. Aside from the fact that this inactivating enzyme accompanies proteolytic enzymes, there is no evidence adduced here that the oxytocic hormone contains peptide linkages.

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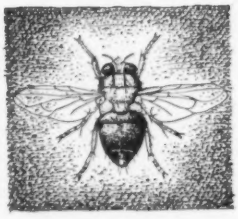

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1. Lister, J.: On the germ theory of putrefaction and other fermentative changes. Nature *8*:232 (July 17) 1873. 2. Gulland, J. M., and Macrae, T. F.: The oxytocic hormone of the posterior lobe of the pituitary gland. IV. The action of preparations of animal proteolytic enzymes, and some observations on the nature of the hormone. Biochem. J. *27*:1383, 1933. 3. Levenbook, L.: Mitochondria of insect flight muscle. J. Histochem. *1*:242 (July) 1953.

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1. Wood, F. C., Gurin, S., and Kuo, P. T.: Medical Correlation Clinic on Atherosclerosis and Coronary Artery Disease, Am. Pract.-Dig. Treat. *12*:235 (April) 1961.

2. Heiskell, C. L., Fisk, R. T., Florsheim, W. H., Yachi, A., Goodman, J. R., and Carpenter, C. M.: A Simple Method for Quantitation of Serum Beta-Lipoproteins by Means of the Immunocrit, Amer. J. Clin. Path. *35*:222 (March) 1961.

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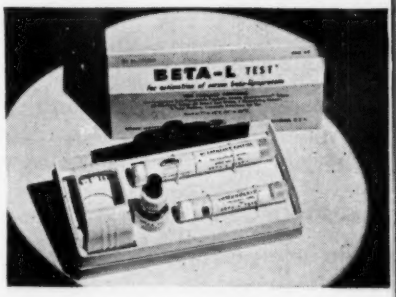

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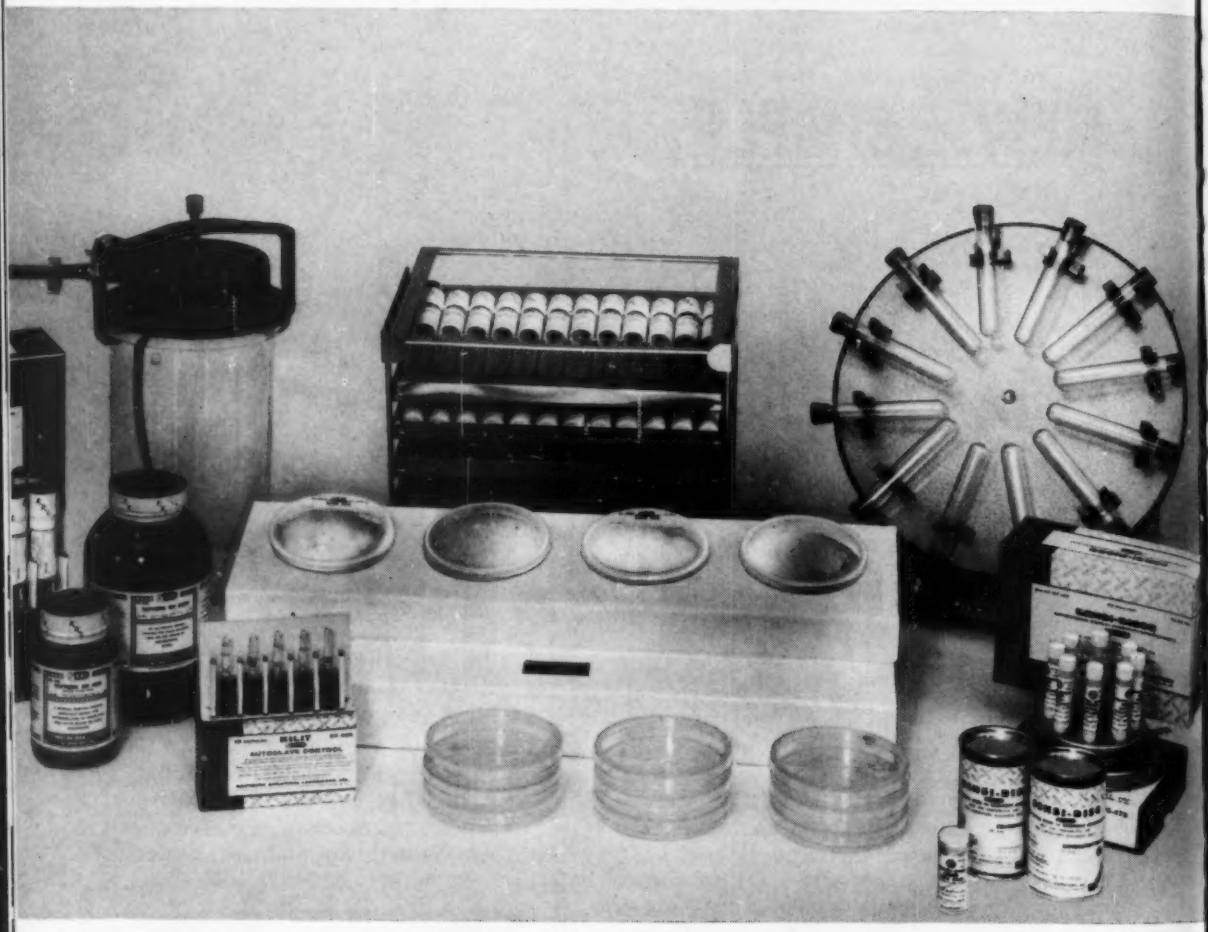

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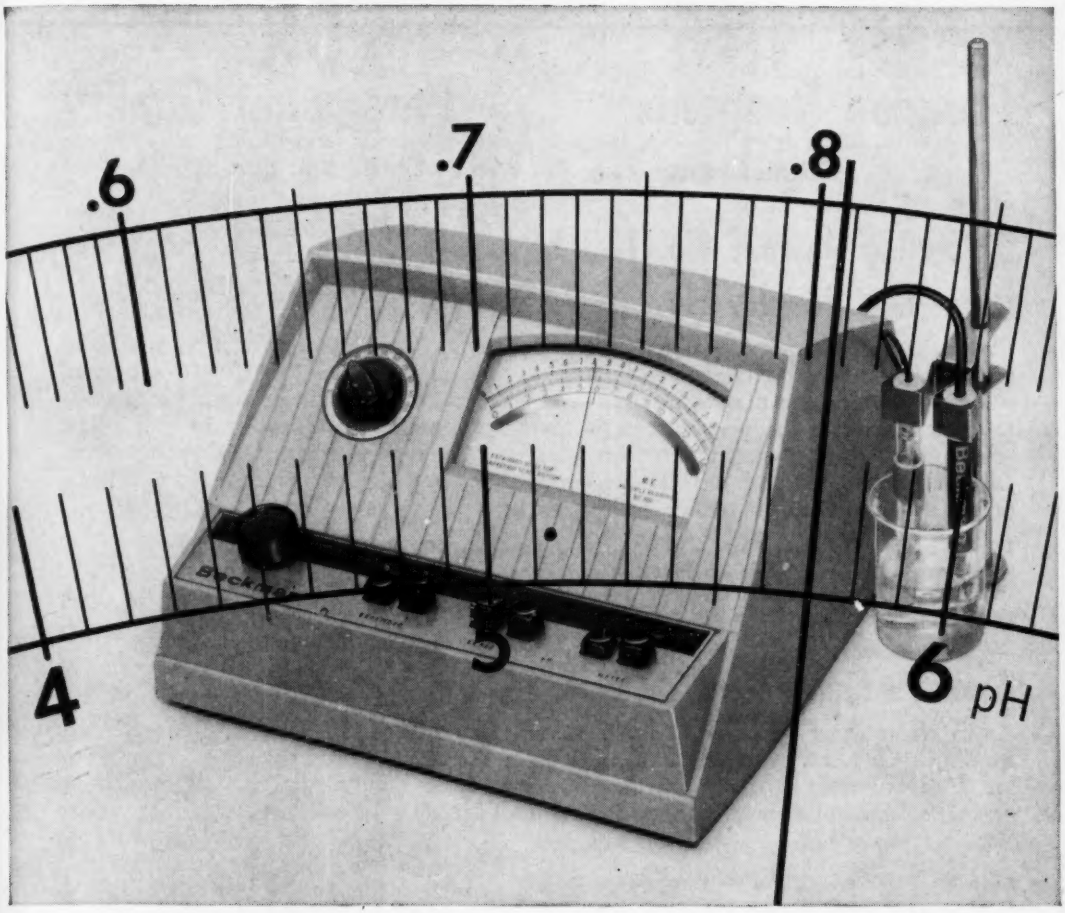

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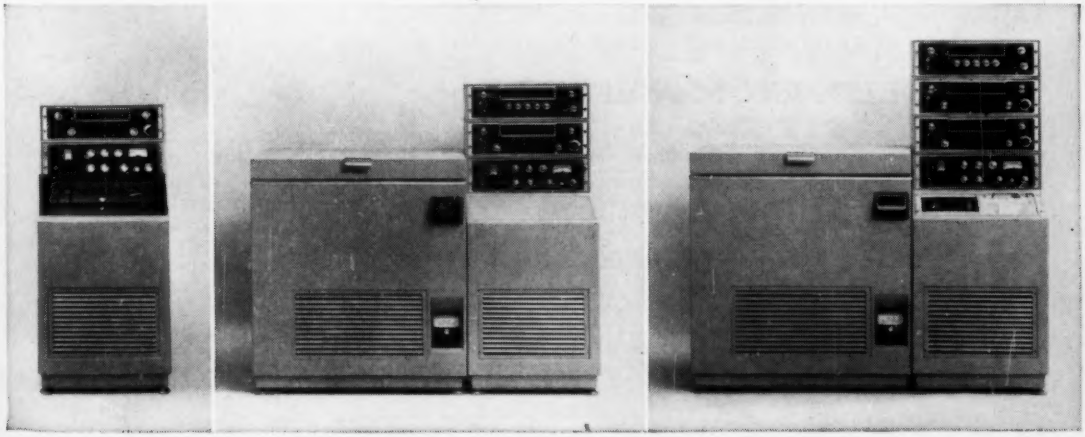

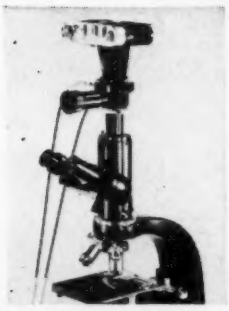

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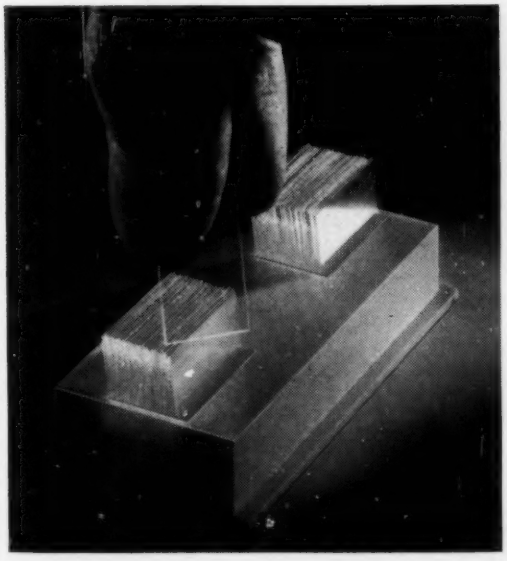

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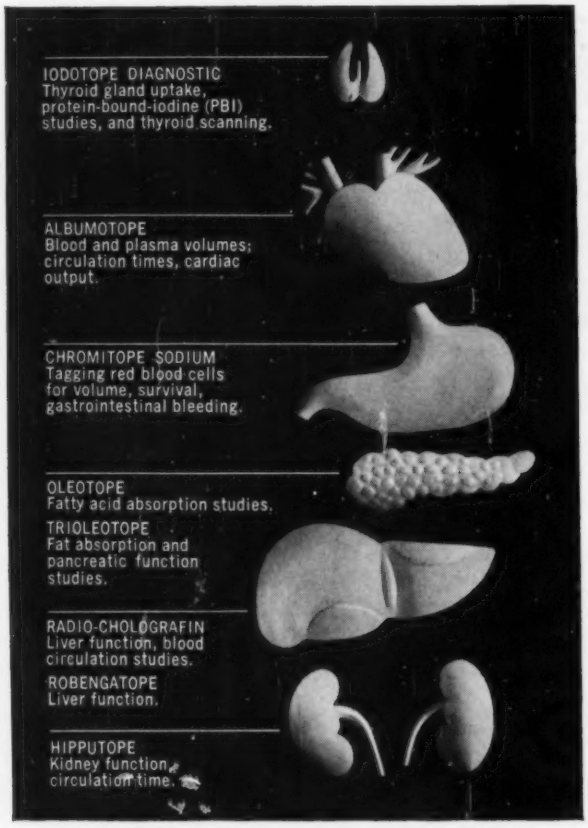

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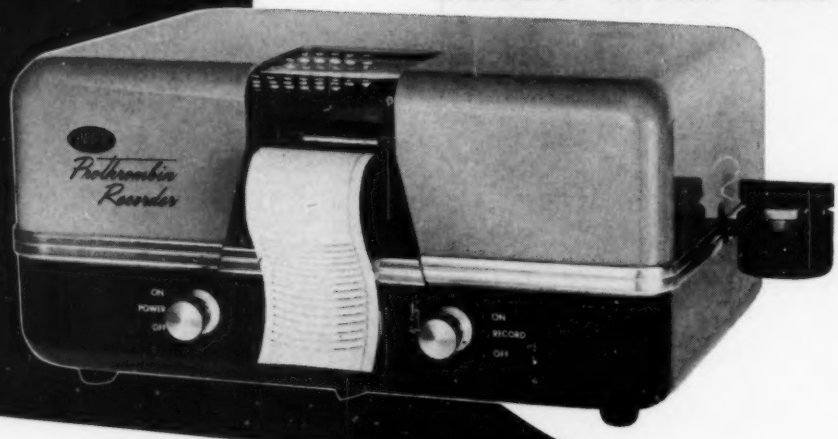

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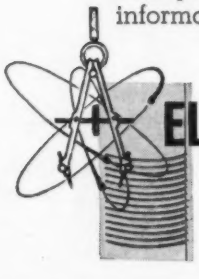

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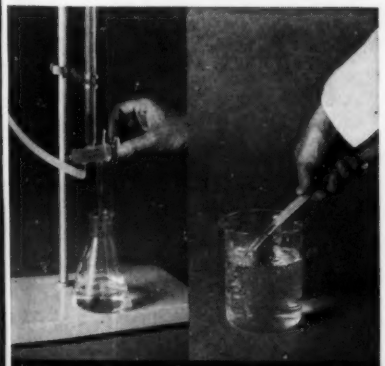

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Clinical and experimental

Starch gel electrophoresis of serum proteins and urinary proteins from patients with multiple myeloma, macroglobulinemia, and other forms of dysproteinemia

Demonstration of diversity and complexity of protein alterations in 72 cases

RALPH L. ENGLE, JR., M.D., KENNETH R. WOODS, PH.D.,
GERMAN B. CASTILLO, M.D.,* and JAMES H. PERT, M.D. *New York, N. Y.*

The application of starch gel electrophoresis to the study of the proteins in the blood and urine of 56 patients with multiple myeloma, 3 patients possibly in the early stages of multiple myeloma, 5 with macroglobulinemia, and 8 others with abnormal peaks in the electrophoretic patterns is described. This method of electrophoresis gives high resolution of protein components and allows a more detailed assessment of protein alterations than can be attained by other methods. It has been possible to demonstrate that the protein abnormality in multiple myeloma is frequently more complex than previously recognized. In the serum patterns usually a single abnormal peak was found, but in 8 cases, 2 were seen; in 3 cases, 3; and in 1 case, 4 peaks were seen. Among the urinary Bence-Jones protein patterns, in 7 cases, 4 bands were seen; in 10 cases, 3; in 12 cases, 2; and in 9 cases, 1 band was seen. There was also some evidence for heterogeneity in the latter 9 cases. Results were compared with those obtained by other methods of electrophoresis, particularly filter paper, and the advantages and disadvantages of the starch gel method are discussed. For routine diagnostic purposes, when only one method is used, the starch gel has no great advantage over filter paper. However, as a research tool in the study of multiple myeloma and related diseases, the starch gel method has some important advantages.

From the Department of Medicine, New York Hospital, Cornell Medical Center, New York, N. Y.
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Received for publication July 1, 1960.
*Fellow of the China Medical Board of New York, Inc.

Although serum and urinary proteins are altered in many diseases, none of the changes are as well documented as those in multiple myeloma. Putnam[2] has reviewed what has been learned about the protein abnormalities in this disease from a variety of methods, including, in part, moving boundary electrophoresis and the relatively simpler filter paper electrophoresis. The present work describes the application of starch gel electrophoresis to the study of the proteins in the blood and urine of 72 patients with multiple myeloma and other dysproteinemias. This method of electrophoresis, originally described by Smithies,[3] gives high resolution and is capable of separating the proteins of normal human serum into over 20 fractions. Hence, the method used in this study allows a more detailed assessment of protein alterations than can be attained by other methods. It has been possible to demonstrate that the protein abnormality in multiple myeloma is frequently more complex than previously recognized. Furthermore, a clearer differentiation of the abnormal proteins has been obtained by virtue of differences in their relative electrophoretic migrations in starch gel.

Methods

Preparation of samples. Blood was drawn from the antecubital vein, transferred to a test tube, and allowed to clot. The serum was either removed and examined during the same day or frozen until the electrophoresis could be performed. Urine was collected under toluene and was examined directly. In some samples the protein concentration was low and better results were obtained after concentration by dialysis against 10 volumes of 4 per cent polyvinylpyrrolidone (PVP). In other instances the urinary proteins were precipitated by two-thirds saturation with ammonium sulfate. The precipitates were collected, dialyzed against distilled water, lyophilized, and applied to the gel as a 2 per cent solution in 0.03 M sodium borate buffer, pH 9.05.

Identification of Bence-Jones proteins in the urine. Bence-Jones proteins were identified by the heat test. In many cases the procedure of Jacobson and Milner[4] was used. The presence of Bence-Jones proteins was confirmed in all patients by electrophoresis and also, in most instances, by salting out and analysis in the ultracentrifuge.

Electrophoresis. The method of starch gel electrophoresis has been described in detail.[3, 5, 6] In summary, soluble starch was produced by acetone-hydrochloric acid treatment of potato starch at 36.1° C. for 70 minutes. Gels were prepared from this soluble starch in 0.030 M sodium borate buffer of pH 9.05 and electrophoresis was carried out with a potential of 4.5 v. per centimeter. The gel strips were stained with naphthalene black B 200. During the course of these experiments several variations in technique were utilized. Some of the samples were run for 5 to 6 hours at room temperature, and others for 16 hours at 10° C. Usually, the samples were inserted into the gel by suspending starch granules in the test serum and transferring an aliquot of the resulting slurry to a prepared slot. Occasionally, filter paper was saturated with the test serum and inserted into an incision in the gel. In some, blood and urine from the same patient were run side by side in the same gel. Terminology of Smithies[3] has been used in the description of bands. Filter paper zone electrophoresis was performed using the method of Flynn and de Mayo[7] with veronal buffer, pH 8.6, ionic strength 0.05. Moving boundary electrophoresis was performed with an Aminco-Stern apparatus using sodium barbiturate buffer, pH 8.6, ionic strength 0.1. All zone electrophoretic patterns were evaluated by visual inspection.

Selection of patients. Samples from 56 patients with multiple myeloma and 5 with macroglobulinemia were examined. In addition, the results of electrophoresis of serum proteins of 8 patients who did not have multiple myeloma or macroglobulinemia and of 3 who may

have been in the early stages of these disorders are presented because of the abnormal patterns obtained.

Diagnosis of multiple myeloma was made upon evidence that there was a plasma cell proliferation, usually invading the bone marrow, associated with the presence of the so-called myeloma proteins in the blood, and/or the presence of Bence-Jones proteins in the urine. In every instance tested but one, the Bence-Jones proteins gave a positive heat reaction. Because of its failure to redissolve upon boiling, the protein in the one exception, Case 15, was classified as "pseudo–Bence-Jones protein."[4] In other respects, including sedimentation coefficient, amino acid composition, and immunochemical behavior, this protein had the characteristics of a Bence-Jones protein. In a few of these cases recognizable plasma cells were not the primary type in the marrow. In one patient the predominating cells were lymphocytes, and in another, they resembled reticulum cells.

In 5 cases the diagnosis of macroglobulinemia was made, based on agar gel diffusion patterns characteristic of increased amounts of gamma globulin of high molecular weight in the blood, a 19 S component. In all 5, the presence of increased amounts of macroglobulin in the serum was demonstrated by the Ouchterlony immunologic method[8] and, in 3, this was confirmed in the analytical ultracentrifuge. Only 3 of these patients had the clinical manifestations of Waldenström's macroglobulinemia.[9]

Results

Table I records the results of starch gel and filter paper electrophoresis of serum and urinary proteins of the patients with multiple myeloma. Of these 56 patients, 13 were found to have an abnormal peak in the serum pattern but no Bence-Jones protein in the urine. On the other hand, 9 had typical Bence-Jones protein peaks in the urinary patterns without having abnormal peaks in the serum patterns. Thirty-one had abnormal peaks in both urine and serum patterns, and one patient with massive diffuse proliferation of myeloma cells in the marrow and with hypogammaglobulinemia had neither. In 2 cases with serum abnormalities, the urine was not tested. Fifty-two cases had demonstrable alterations in serum pattern even though abnormal peaks might not have been evident.

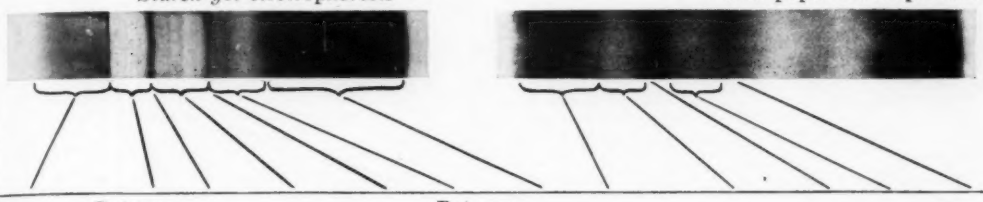
The distribution of the abnormal peaks as determined by starch gel zone electrophoresis of serum and urinary proteins is summarized in Table II. The abnormal peaks in the serum pattern occurred somewhere between the slow end of the gamma globulin region and the region just slower than albumin; frequently they were sharp, indicating a high degree of homogeneity, but sometimes they were diffuse (Fig. 1). Usually a single abnormal peak was found, but in 8 cases, 2 were seen; in 3 cases, 3; and in 1 case, 4 peaks were seen (Figs. 2, 3, and 4). When multiple abnormal peaks were seen, in some instances there was an apparent periodicity in the placement of the peaks, while in others the peaks appeared in widely and irregularly separated regions of the gel. In one instance, freezing the plasma seemed to result in a diminished resolution and a slight alteration in mobility (Fig. 4). Generally, freezing was not observed to produce any notable alteration in gel electrophoretic properties. In another instance a diffuse myeloma peak in the region of slow $alpha_2$ globulin covered the normal slow $alpha_2$ band (Fig. 3). In rare cases the myeloma peak appeared to be an elevation of a normal peak, for example, see Case 32, Fig. 1, wherein an elevation of the slow $alpha_2$ band is seen.

Table I. Results of starch gel and filter paper electrophoresis of serum and urinary proteins from patients with multiple myeloma

Patient No.	*History No. or initials*	*Age*	*Sex*	*Sample†*	*Serum proteins, albumin/globulin (Gm. %)*	*Bence-Jones protein*
Patients with gamma serum myeloma peaks on starch gel electrophoresis.						
1	654,885	59	F	S	4.4/2.8	
				U‡		+
2	J. D.	77	M	S		
				U		
3	673,352	40	M	S	1.9/11.8	
				U§		+
4	662,470	49	F	S	1.9/12.0	
				U¶		−
5	663,526	55	M	S	3.1/7.0	
				U		+
6	713,989	66	F	S	4.2/5.3	
				U		−
7	362,432	48	M	S	3.3/0.8	
				U¶		+
8	G. K.	59	M	S	3.6/9.3	
				U§		+
9	L. L.	68	F	S		
				U		−
10	669,033	61	F	S	3.1/7.0	
				U¶		+
11	780,368	57	M	S	2.2/7.8	
				U		−
12	J. R.	63	M	S	1.5/14.0	
				U		−
13	713,090	38	F	S	4.7/3.8	
				U		−
14	733,685	61	F	S	3.6/2.4	
				U		+
15	601,298	65	M	S	4.0/1.7	
				U§		−
16	646,765	41	M	S	4.2/3.4	
				U		−
17	F. K.	50	F	S	3.8/4.3	
				U		−
18	790,493	53	F	S	2.2/7.9	
				U		+
19	317,080	65	F	S		
				U		

*Mobility and number of abnormal peaks and amount of normal gamma globulin**

Starch gel electrophoresis | *Filter paper electrophoresis*

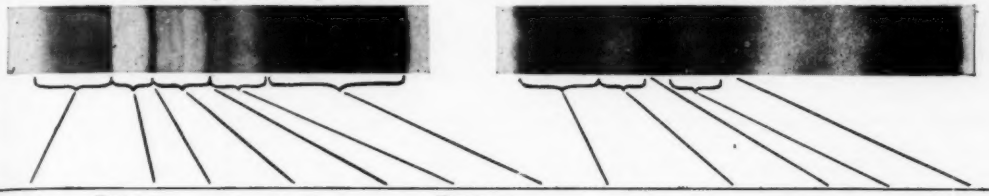


Normal γ	*γ*	*Between origin and Sα₂*	*Slow α₂*	*Between Sα₂ and β*	*β*	*Between β and albumin*	*Albumin*	*Normal γ*	*γ*	*Between β and γ*	*β*	*Between α₂ and β*	*α₂*
d	2							d	1				
		Inadequate resolution							4				
	1							d	1				
				3					1				
d	1							d	1				
				4					2				
d	1							d	1				
		No peaks							No peaks				
d	1								1				
										1			
d	1							d	1				
d	1							d					
	2								1				
d	1								1				
						3					1		
d	1								1				
d	1							d	1				
						2			1		1		
d	2								1				
	2								1				
	1												
d	1												
	1								1				
	3	1											
d	1							d					
		3							1				
d	1							d	1				
d	1								1				
d	2												
	1												
d	4-5												

Table I. Cont'd

Patient No.	*History No. or initials*	*Age*	*Sex*	*Sample†*	*Serum proteins, albumin/globulin (Gm. %)*	*Bence-Jones protein*	*Normal γ*
20	688,185	69	M	S	3.5/5.2		d
				U		–	
21	805,564	64	F	S	2.0/9.8		d
				U		–	
22	811,773	76	M	S	3.7/4.3		d
				U			
23	812,745	61	M	S	2.4/5.9		
				U			
24	824,243	58	M	S	3.4/2.6		n
				U			
25	754,604	82	M	S	4.1/5.5		d
				U			
Patients with serum myeloma peaks between the origin and slow alpha$_2$ component on starch gel electrophoresis.							
26	683,038	66	M	S	1.9/10.7		d
				U§		+	
27	727,933	68	F	S	3.1/4.6		d
				U§		+	
28	780,966	53	M	S	2.2/8.5		d
				U		–	
29	764,336	52	F	S	3.6/2.5		d
				U§		+	
30	788,045	67	M	S	3.0/5.1		d
				U		+	
Patients with slow alpha$_2$ serum myeloma peaks on starch gel electrophoresis.							
31	S. B.	65	M	S	4.2/1.2		d
				U§		+	
32	744,826	58	F	S	2.0/2.1		d
				U		+	
33	38,237	58	F	S	3.8/3.7		d
				U§		+	
34	757,267	39	M	S	2.4/6.4		d
				U§		+	
35	783,682	72	M	S	3.1/7.0		d
				U		+	
36	784,829	61	M	S	3.4/4.5		d
				U¶		+	

*Mobility and number of abnormal peaks and amount of normal gamma globulin**

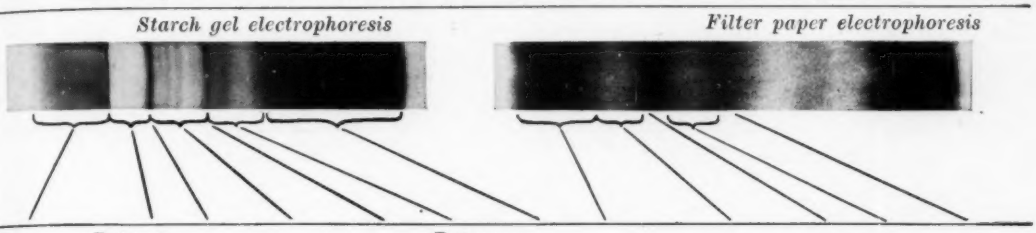


ce-Jones rotein	Normal γ	γ	Between origin and $S\alpha_2$	Slow α_2	Between $S\alpha_2$ and β	β	Between β and albumin	Albumin	Normal γ	γ	Between β and γ	β	Between α_2 and β	α_1
–	d	3							d	1				
–	d	1			3				d		1			
·	d	1							d	1				
·		1												
		2												
·		2												
·	n	3												
	d	1												
+	d		2				3		d	1				
+	d		1				2		d	1 (?)		1		
–	d		1								1			
+	d		2											
+	d		1	3										
					2		2				1			
+	d			i					n					
+					2						1			
+	d			i				1	d					
+	d			1			1		d		1			i
+	d			1								1		1
+			3											
+	d	2		1					d		2			
		4												
+	d			1			2				1			

Table I. Cont'd

Patient No.	*History No. or initials*	*Age*	*Sex*	*Sample†*	*Serum proteins, albumin/globulin (Gm. %)*	*Bence-Jones protein*
Patients with serum myeloma peaks between slow alpha$_2$ and albumin component in starch gel electrophoresis.						
37	J. O.	81	M	S	3.5/3.4	
				U		
38	D. C.	58	F	S	3.2/6.1	
				U		–
39	M. R.	59	F	S	2.1/4.0	
				U		–
40	658,468	65	M	S	4.5/1.5	
				U§		–
Patients without demonstrable serum myeloma peaks but with hypogammaglobulinemia on starch gel electrophoresis.						
41	654,371	38	M	S	4.1/1.3	
				U§		+
42	765,568	52	F	S	1.1/9.8	
				U§		+
43	61,235	66	M	S	4.2/1.4	
				U		+
44	O. J.			S		
				U		+
45	699,507	51	M	S	5.3/1.8	
				U		–
46	233,456	48	F	S	4.4/1.7	
				U		+
47	811,605	58	F	S	2.3/8.1	
				U		+
48	790,571	71	M	S		
				U		+
49	775,635	72	F	S	3.6/2.0	
				U		+
Patients without demonstrable serum myeloma peaks and with normal gamma globulins.						
50	675,248	52	M	S	5.4/2.4	
				U§		–
51	742,613	61	M	S	4.1/1.9	
				U§		–
52	151,204	69	F	S	3.9/1.5	
				U		+
53	812,529	78	M	S	3.6/1.9	
				U		–

*Mobility and number of abnormal peaks and amount of normal gamma globulin**



Jones protein	Normal γ	γ	Between origin and $S\alpha_2$	Slow α_2	Between $S\alpha_2$ and β	β	Between β and albumin	Albumin	Normal γ	γ	Between β and γ	β	Between α_2 and β	α_2
+	d				2				d			1		
+	d					1								
−	d					1			d				1	
−	d						1		d				1	
−							2						1	
+	d		No abnormal peaks						d	No abnormal peaks				
+	d		No abnormal peaks				3					1		
+	d		No abnormal peaks		1				d		1			
+	d		No abnormal peaks				1							
+	d		No abnormal peaks				2							
−	d		No abnormal peaks						d		No abnormal peaks			
+	d		No abnormal peaks						d		No abnormal peaks			
+	d	1	No abnormal peaks						d	1				
+	d		No abnormal peaks		3		1							
+	d		No abnormal peaks		3									
+	d		No abnormal peaks					1						
+	n		No abnormal peaks						n	(?) 1				
−		2	1		2					1				
−	n		No abnormal peaks						n		No abnormal peaks			
+		2	1		1					1				
+	n		No abnormal peaks		2									
+	n		No abnormal peaks				4		n					i

Table I. Cont'd

Patient No.	*History No. or initials*	*Age*	*Sex*	*Sample†*	*Serum proteins, albumin globulin (Gm. %)*	*Bence-Jones protein*
Patients in whom starch gel electrophoresis of serum proteins was not performed.						
54	641,679	55	M	S	3.9/1.6	-
				U§		+
55	733,408	58	M	S	4.3/3.0	
				U‡		+
56	416,092	23	F	S	1.2/13.0	
				U§		+

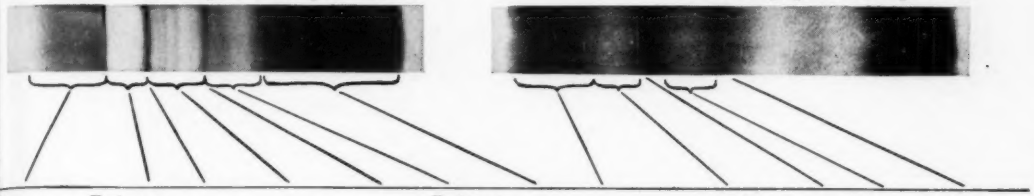
*n = normal, d = decreased, i = increased.
†S = serum, U = urine.
‡Electrophoresis of urine and purified protein in starch gel gave poor resolutions.
§Urinary protein precipitated with $(NH_4)_2 SO_4$.
¶Urine concentrated.

A visual evaluation indicated that albumin was decreased in almost all instances and that the gamma globulins in areas not covered by the myeloma peaks were decreased in 44 cases. While it is sometimes difficult to evaluate the concentration of gamma globulin from visual inspection of the electrophoretic patterns, in all of these cases the deficiency was thought to be a definite one. Some changes in many of the other protein components were difficult to distinguish from normal variants. For example, in some instances additional bands were interpreted as splitting of the slow $alpha_2$ or beta globulin bands, the latter phenomenon having been shown to be genetically determined.[10] Concentrations of some of the other components varied, but these were usually difficult to evaluate.

Some of the electrophoretic patterns of the urinary proteins are illustrated in Fig. 5. The typical Bence-Jones protein peaks in the urinary patterns were discrete and migrated in the gel anywhere from the slow end of the gamma globulin region to that region just slower than albumin. In 7 instances, 4 bands were seen; in 10 instances, 3; in 12 instances, 2; and in 9 instances, 1 band. These latter specimens seemed to be homogeneous under conditions of the analysis. However, further study of one of the proteins revealed that 2 bands could be resolved by modifying the conditions of electrophoresis. It is quite possible that the others are also heterogeneous. In 26 of the cases the bands occurred close to each other with about the width of a band between them. The band nearest the cathode was always the most intense. There were 3 cases wherein 4 bands were seen to occur in widely separated portions of the gel.

*Mobility and number of abnormal peaks and amount of normal gamma globulin**

Starch gel electrophoresis *Filter paper electrophoresis*



	Between origin and $S\alpha_2$	*Slow α_2*	*Between $S\alpha_2$ and β*	*β*	*Between β and albumin*	*Albumin*	*Normal γ*		*Between β and γ*	*β*	*Between α_2 and β*	*α_2*
							d	1				
+		2						1				
							d			1		
+	Inadequate resolution								1			
							n		1			
+			2									

In most instances where both serum myeloma proteins and urinary Bence-Jones proteins were encountered in the same patient, the mobilities of the 2 proteins differed from each other, the urinary protein usually migrating faster than the serum myeloma protein. However, in 2 instances where 2 peaks were identified in the serum, one of them had about the same mobility as the urinary Bence-Jones protein from the same patient. In still another case with Bence-Jones proteinuria, there was a mid-gamma peak of almost identical mobility in both serum and urine. These cases suggest that occasionally there might be a Bence-Jones proteinemia of significant degree (Fig. 2). In addition to the Bence-Jones protein, many urine samples contained relatively small amounts of other serum proteins, such as albumin and beta globulin. There was usually no difficulty, however, in identifying the components that differed from those seen in the serum.

Also considered here are the sera from 5 patients with macroglobulinemia, 4 of which were examined with the starch gel method. In one case with 3 Gm. per cent macroglobulin in the serum (estimated from ultracentrifuge pattern) no unusual band which could account for the expected quantity of abnormal protein was encountered in the electrophoretic pattern (Fig. 6). Considerable protein remained at the origin and did not enter the gel. The gamma globulin was decreased, beta globulin and the haptoglobins were increased, and a moderately intense band was present between beta globulin and albumin. Other components were normal. Two cases had marked decrease of the gamma globulins and no evidence of a macroglobulin band. In the fourth case, a generalized hypergammaglobulinemia was observed. In 2

Table II. Distribution of abnormal peaks as determined by starch gel electrophoresis of serum and urinary proteins (multiple myeloma patients only)

Mobility and No. of abnormal peaks	*No. of patients*	
	Serum	*Urine*
Gamma		
1 peak	17	4
2 peaks	5	2
3 peaks	2	0
4 peaks	1	2
Between origin and slow $alpha_2$		
1 peak	3	0
2 peaks	2	1
3 peaks	0	2
Slow $alpha_2$		
1 peak	2	0
2 peaks	0	1
3 peaks	0	1
Slow $alpha_2$ + gamma		
3 peaks	4	0
Between slow $alpha_2$ and beta		
1 peak	0	1
2 peaks	1	3
3 peaks	0	4
4 peaks	0	1
Beta		
1 peak	2	0
Between beta and albumin		
1 peak	1	2
2 peaks	0	5
3 peaks	0	3
4 peaks	0	1
Four bands in more than 2 regions	0	3
Albumin migration but Bence-Jones heat test positive	0	2
Hypogammaglobulinemia only (no myeloma peak)	9	0
No significant abnormality	4	6
Total	53	44

of the 5 cases of macroglobulinemia, there was Bence-Jones protein in the urine. Starch gel electrophoresis of one of these urine samples revealed a single band in the slow gamma region.

Eight patients who probably do not have multiple myeloma or macroglobulinemia are mentioned because their sera showed the presence of abnormal peaks in the starch gel electrophoretic patterns. Diagnoses in this group included hypersensitivity to cold, emphysema and gastrointestinal bleeding, tuberculosis and liver disease, periodic hypervolemic shock (patient reported by Clarkson and associates[11]), granulocytic leukemia, diabetes with hypertensive cardiovascular

disease and goiter, coronary occlusion and bleeding tendency, and hypercorticism syndrome. In most of these patients there appeared to be an obscure underlying process which could not be adequately defined. In 3 of these patients the starch gel electrophoretic pattern revealed a discrete gamma peak superimposed on a normal gamma globulin (Figs. 7 and 8). This contrasted with most of the cases of multiple myeloma where a markedly decreased normal

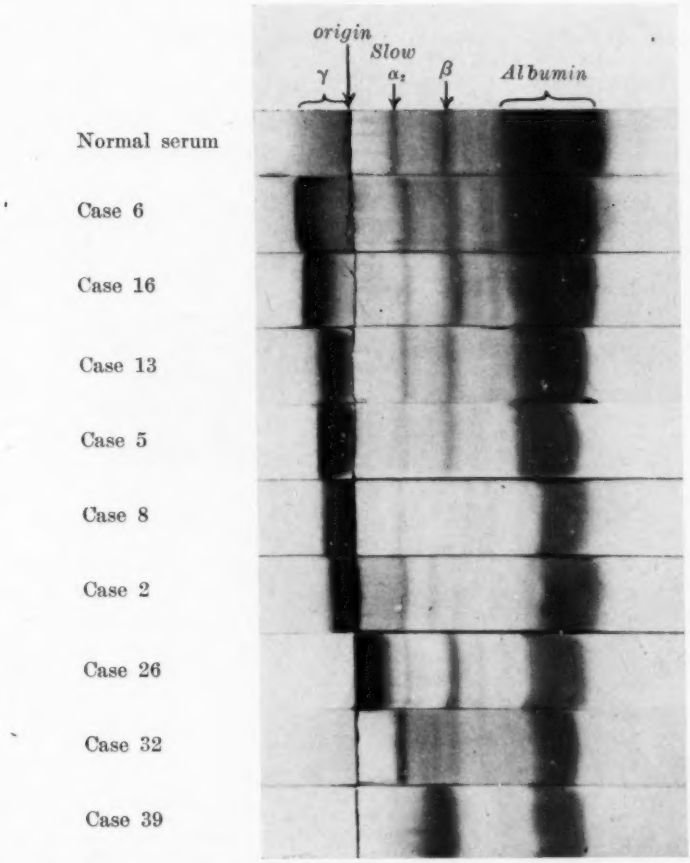


Fig. 1. Gel electrophoresis of serum proteins from several patients with multiple myeloma showing variability in migration of myeloma peak. (In all illustrations showing electrophoretic patterns the origin is marked with an arrow, the anode is toward the right, and the cathode is toward the left. Each photograph is a composite of several individual patterns. This makes it difficult to compare exact mobilities.)

gamma globulin component was evident. Three patients had 2 discrete gamma peaks superimposed on a normal gamma globulin, and 2 had a discrete gamma globulin peak associated with a decrease in the normal gamma globulin. None of these patients had any Bence-Jones protein in the urine.

Three additional patients had no manifestations of multiple myeloma except for abnormal serum patterns, but these patients may eventually develop

other manifestations of the disease (Fig. 9). Bleeding was evident in 2 of these patients, one with a fast gamma peak and hypogammaglobulinemia, the other with a serum protein pattern that was normal on starch gel electrophoresis but that showed a gamma peak on filter paper electrophoresis. The third patient had the menopausal syndrome, and the serum gel electrophoretic pattern showed a discrete gamma peak superimposed on a normal gamma globulin.

Comparison of starch gel electrophoresis with other methods

When the patterns derived from starch gel electrophoresis of the serum and urinary proteins were compared with those obtained by filter paper electrophoresis, the mobilities of the abnormal serum proteins showed good correlation

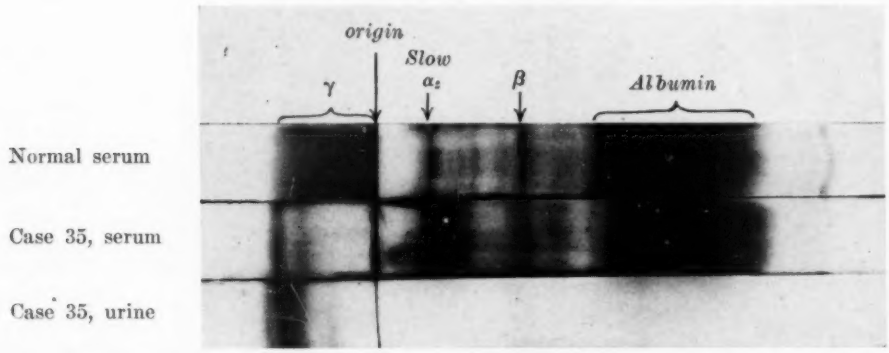


Fig. 2. Gel electrophoresis of serum and urine from same patient with multiple myeloma. Serum pattern shows broad band in region of slow apha$_2$ globulin and 2 discrete bands in the gamma globulin region. The urinary pattern shows 3 bands in the gamma globulin region, one of which corresponds to the same mobility as one of the bands in the serum pattern. Three additional bands, probably normal serum proteins, are seen in the anodal end of the gel.

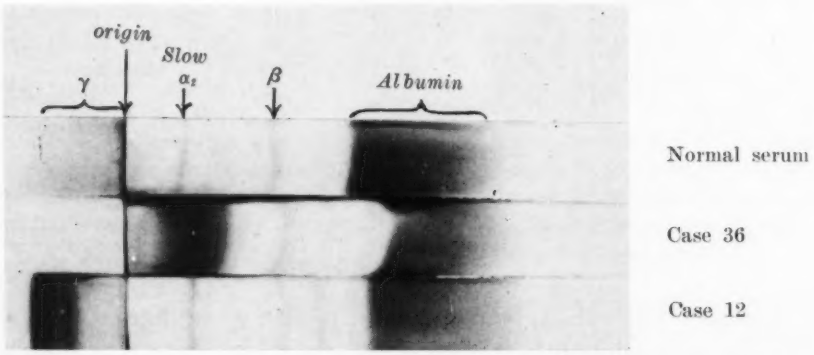


Fig. 3. Gel electrophoresis of serum proteins from 2 patients with multiple myeloma. The strip from Case 36 reveals hypogammaglobulinemia and the presence of a broad myeloma peak in the region of slow alpha$_2$ globulin. The normal slow alpha$_2$ globulin band can be seen superimposed on the abnormal peak. The strip from Case 12 reveals 2 abnormal components in the region of gamma globulin. (Photograph has been purposely overexposed in order to demonstrate the multiple components. Other bands are thereby made less intense.)

(Table I). Where exceptions were noted, it was not determined whether effects of the supporting medium or of the buffer composition were responsible.

In comparing the patterns obtained by starch gel electrophoresis with those obtained by other methods, certain differences were noted. In one patient with hypersensitivity to cold, there was a definite gamma peak superimposed on the normal gamma pattern in the starch gel electrophoretic pattern while only a hypergammaglobulinemia could be seen in the filter paper and moving boundary electrophoretic patterns. In several instances where more than one peak was evident on gel, only one myeloma peak was seen on filter paper. Conversely, in three instances of multiple myeloma without macroglobulinemia, no myeloma peaks were demonstrable in the gel electrophoresis pattern of sera, whereas peaks were found either on paper electrophoresis, on moving boundary electrophoresis, or on both. Furthermore, in no case of macroglobulinemia were serum myeloma peaks seen, probably because the gamma macroglobulin did not migrate into the gel. Bence-Jones proteins almost always resolved into

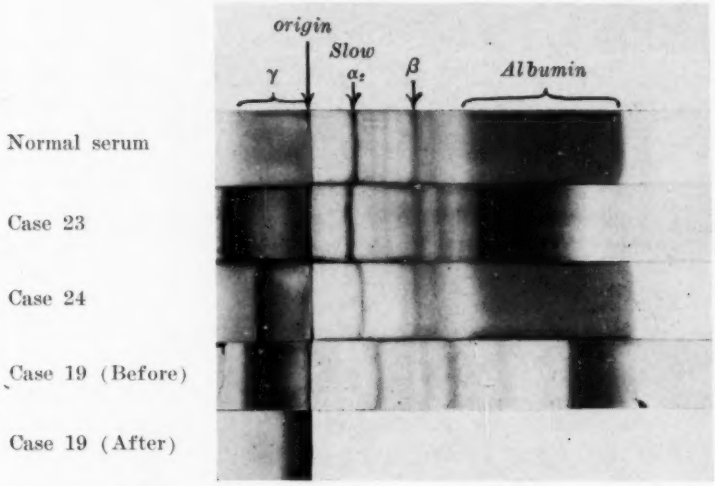


Fig. 4. Gel electrophoresis of serum proteins of 3 patients with multiple myeloma showing multiple abnormal peaks in each pattern. In one instance, Case 19, the change in pattern produced by freezing the serum is shown. Note change in resolution and mobility of protein in frozen sample. (Only proteins migrating toward cathode are shown in the last pattern; the other portion of the pattern was normal.)

more components on gel than could be demonstrated on paper, where only one component was usually seen. In one striking exception, 4 gamma peaks were seen on paper electrophoresis. While most Bence-Jones proteins migrated in discrete bands, in 2 instances, even though large amounts of lyophilized proteins were applied, the protein did not resolve on the starch gel, and only very faint indistinct bands appeared. The reason for this is not clear. It was possible to get good resolution of these proteins upon filter paper electrophoresis.

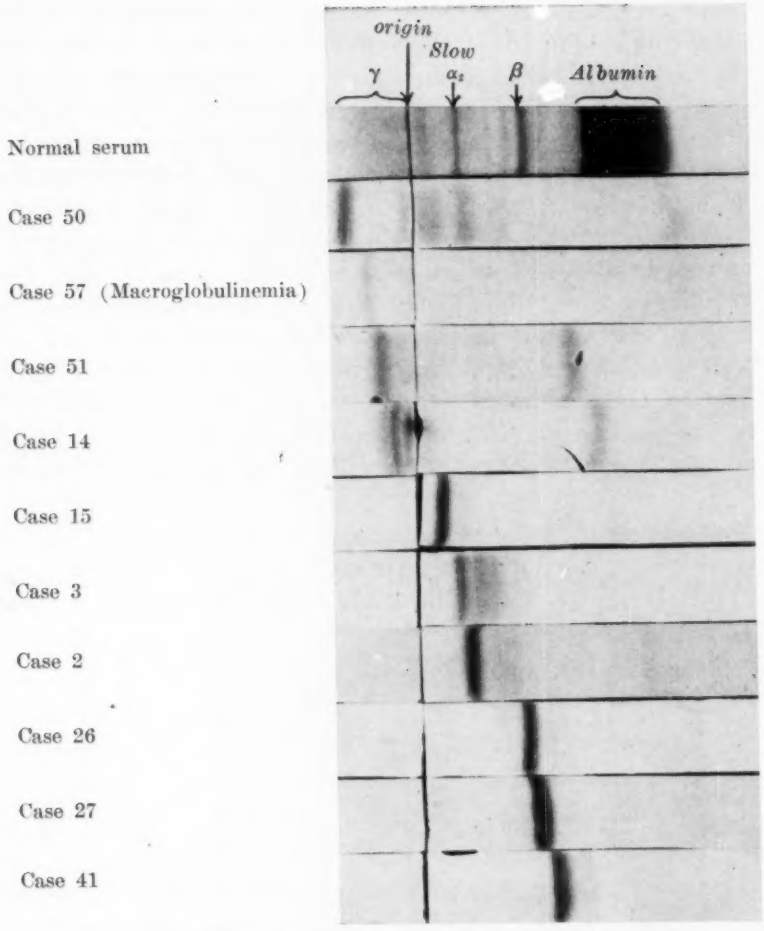


Fig. 5. Gel electrophoresis of urinary Bence-Jones proteins from patients with multiple myeloma. Note heterogeneity of samples and variation in mobilities. An albumin band is noted in some samples. Not all of the Bence-Jones protein bands are visible.

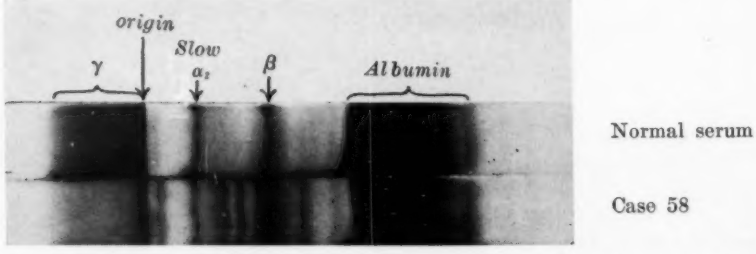


Fig. 6. Gel electrophoresis of serum proteins from a patient with macroglobulinemia, 3 Gm. per cent. Note the hypogammaglobulinemia. There is no peak comparable to the large amount of macroglobulin which was visible upon filter paper electrophoresis.

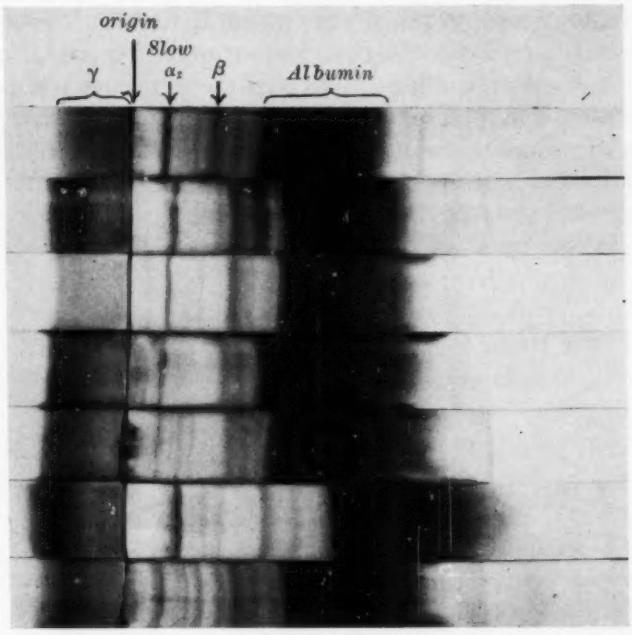


Fig. 7. Gel electrophoresis patterns of serum from patients who probably do not have multiple myeloma. Note one or more spikes in gamma globulin region, usually superimposed on a normal gamma globulin pattern.

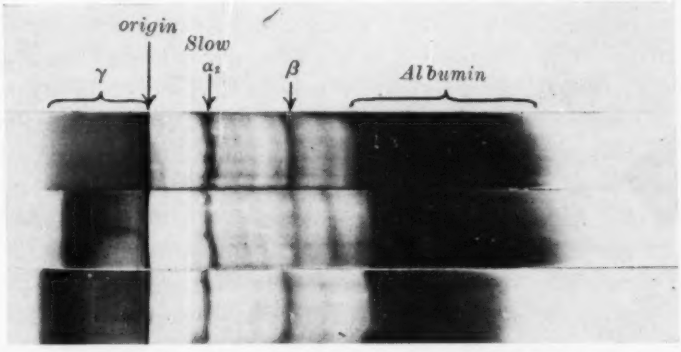


Fig. 8. Gel electrophoretic patterns of serum from patients who probably do not have multiple myeloma. Note one or more spikes in gamma globulin region, usually superimposed on a normal gamma globulin pattern.

Discussion

Almost all patients with multiple myeloma have abnormal protein peaks in the electrophoretic patterns of the serum and/or urine as determined by moving boundary[12] and filter paper electrophoresis.[13] The mobilities of the abnormal peaks in both serum and urine differ from patient to patient and the peaks may be found to migrate at any point from the slow end of the gamma

globulin region to the alpha$_2$ globulin region. Observations emphasizing the diversity of physicochemical properties among proteins derived from different patients with this disease have been the subject of a series of reports by Putnam; his work and that of others has been summarized in a review article.[14] The clinical aspects of multiple myeloma have been reviewed by Osserman.[15]

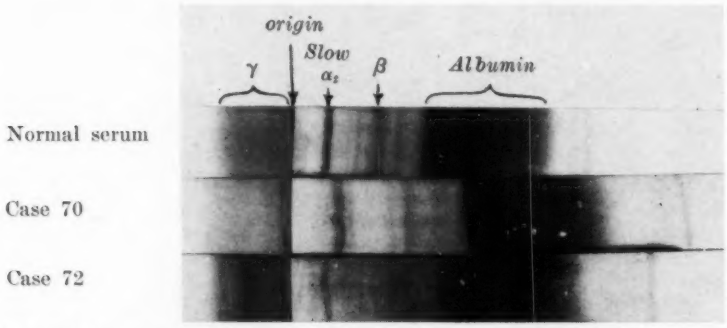


Fig. 9. Gel electrophoretic patterns of serum proteins of 2 patients who do not as yet have other manifestations of multiple myeloma. Note peaks in gamma region.

Although it is not certain, the serum myeloma proteins and the urinary Bence-Jones proteins are thought to be derived from the abnormal plasma cells which are characteristic of the disease. The Bence-Jones proteins have much smaller molecular weights than do the serum myeloma proteins, and they are cleared very rapidly from the blood by the kidney. The question of whether the abnormal protein peaks seen in the blood of patients with multiple myeloma result from the presence of excessive amounts of a normal constituent or from large amounts of an abnormal or modified protein has never been answered. The fact that the range of mobilities of the myeloma proteins is comparable to the range of mobilities of antibody activity is compatible with either thesis. When both a serum myeloma and urinary Bence-Jones protein are present in the same patient, the mobilities of the 2 proteins are usually different. The abnormal peaks in the serum and/or urine of an individual patient, however, maintain the same mobility throughout the course of the disease. Usually a single abnormal protein component is found upon serum electrophoresis in any one patient. In rare instances 2 or 3 components have been described.[9, 16-19] The electrophoretic heterogeneity of some Bence-Jones proteins has also been recognized, and 2 components have been found in the urine of the same patient by workers using both moving boundary and filter paper electrophoresis,[20, 21] and as many as 3 to 5 components have been recognized by moving boundary electrophoresis.[20]

Starch gel electrophoresis has made it possible to demonstrate a greater degree of heterogeneity of some serum myeloma proteins and most Bence-Jones proteins than heretofore recognized. Since our preliminary report in 1957,[1] which emphasized the heterogeneity of the Bence-Jones proteins, other workers using the same technique have made similar observations.[22, 25] Results of the present study are in agreement with observations on the heterogeneity of some

serum myeloma proteins reported by Flynn and Stow,[22] Creyssel and Fine,[23, 24] Owen, Got, and Silberman,[25] and Fine, Creyssel, and Morel.[26, 27] The multiplicity of bands can be explained either on the basis of differences in degree of aggregation of identical or similar molecules or on the basis of differences in molecular species. Except in the case of macroglobulins, there is little evidence that serum myeloma or Bence-Jones proteins polymerize, and the most likely explanation for heterogeneity is the presence of different, possibly closely related, molecular species.

Since in the majority of cases of multiple myeloma each patient is known to produce at least 2 aberrant proteins, the myeloma protein which appears in the serum and the Bence-Jones protein which is excreted in the urine, the possibility must be considered that heterogeneity in the serum or urinary protein pattern of these patients is related to the occasional excretion of myeloma protein into the urine or retention of Bence-Jones protein in the serum as a result of kidney damage. While there is some evidence for occasional presence of myeloma proteins in the urine and for occasional Bence-Jones proteinemia of appreciable amount,[28-30] in the great majority of cases heterogeneity cannot be explained by these mechanisms.

We have seen several cases wherein filter paper electrophoresis suggested that the myeloma protein in addition to the Bence-Jones protein was being excreted in the urine. In 2 of these patients on whom the studies were repeated in the starch gel, the mobilities were demonstrated to differ, and it is now believed that the 2 bands seen in the filter paper electrophoresis of the urine are both Bence-Jones protein. Flynn and Stow[22] described a patient with multiple myeloma and generalized proteinuria in whom all of the abnormal and normal serum components were also identified in the starch gel patterns of the urinary proteins. They imply that in this case the abnormal bands in the urine are the myeloma proteins, rather than Bence-Jones, since the Bence-Jones heat test was negative.

On the other hand, the electrophoretic patterns of serum and urine from Patients 22, 23, and 35, who did not have an appreciable glomerular leak of protein, suggest that the Bence-Jones protein may be retained in the blood in appreciable amounts. The urine was positive for Bence-Jones protein. However, when the samples of serum and urine were run side by side in the same gel, minor differences in mobility were noted between the serum and urinary components in the first 2 of these cases. In the third case, the serum protein was a pyroglobulin; i.e., it precipitated, and the serum gelled when heated at 63° C. for 5 minutes. Isolation and characterization of the pyroglobulin from the serum demonstrated that it was a myeloma protein and not the Bence-Jones.[31] Flynn and Stow[22] have also described one patient with Bence-Jones proteinuria who did not have an appreciable glomerular leak but who showed an abnormal electrophoretic band common to both serum and urine.

The frequent demonstration of heterogeneity of serum myeloma protein in single serum samples as well as heterogeneity of Bence-Jones protein in single urine samples indicates that the protein abnormality is frequently more complex than previously recognized. This information in itself does not help to answer

the question whether the serum myeloma proteins are normal proteins present in unusually high concentration or are abnormal proteins. However, it does bring up the possibility that in some serum electrophoretic patterns the unusual peaks are both normal and abnormal components comparable to the situation in sickle trait wherein both sickle and normal hemoglobins appear in the same individual. Still another possibility that must be seriously considered is that more than one cell line is involved in the malignant proliferation. The detailed evidence for and against the various theories has recently been reviewed by Owen and Got.[32] However, the answers to these problems must await more detailed information of protein structure, protein synthesis, and genetics.

For routine diagnostic purposes, when only one method is used, the starch gel has no great advantage over filter paper electrophoresis. However, as a research tool in the study of multiple myeloma and related diseases, the former has some important advantages over the latter. The increased resolution points out previously unrecognized heterogeneity in some myeloma serum proteins and Bence-Jones proteins and results in more precise comparisons of relative electrophoretic mobility. Occasionally, discrete peaks not recognized by other methods are seen in the electrophoretic pattern of patients who do not appear to have multiple myeloma. The significance of this is not yet known. Other workers have reported similar observations.[26, 27, 32] The possibility that these discrete peaks represent manifestations of a somatic mutation should be considered.

Disadvantages of the method are the increased time, expense, and skill required, and difficulties in standardizing the gel, in obtaining quantitative measurements of the amount of each fraction obtained, and in obtaining absolute mobilities. Macroglobulins of gamma mobility usually do not migrate into the gel and would be missed. However, the characteristic appearance of a macroglobulin upon filter paper or moving boundary electrophoresis and its absence in gel electrophoresis is strong evidence for macroglobulinemia.[1, 24] Rarely, typical myeloma peaks in the serum and Bence-Jones peaks in the urine are not resolved by the gel, for reasons that are not yet known.

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The effect of Polybrene on blood coagulation. In vitro

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Since Polybrene, a new antiheparin agent, has itself been reported to be an anticoagulant, studies were carried out to determine its in vitro effect on the various stages of blood coagulation. In concentrations comparable to dosages administered clinically, Polybrene prolongs whole blood clotting, probably by interfering with the production of "thromboplastic" activity, thus hindering conversion of prothrombin to thrombin. The degree of Polybrene's inhibition of coagulation is inversely related to the number of platelets present. In the thromboplastin generation test, its inhibition is also counteracted by heparin and serum factors, particularly prothrombin, factor IX, and, to a lesser extent, platelet-like activity of serum, but not by a crude cephalin or by adsorbed plasma. Polybrene is fibrinoplastic in the thrombin-fibrinogen reaction, but does not itself precipitate fibrinogen from plasma. In relatively higher concentrations, it delays the one-stage prothrombin time determination and agglutinates erythrocytes. The clinical implications of these findings are discussed.

In 1952, Preston[1] first reported the antiheparin action of Polybrene,† a quaternary ammonium salt having the empirical formula $(C_{13}H_{30}Br_2N_2)_X$. Since then several accounts of its clinical use in the neutralization of heparin have been published.[2-4]

Preston also showed that, similar to protamine, Polybrene prolongs the clotting time of blood in vitro,[5] but, administered intravenously in doses of 0.5 to 1.0 mg. per kilogram, had no effect on the clotting time in vivo.[6] With considerably higher doses (7.5 to 10 mg. per kilogram), injected intravenously in dogs, prolongation of the clotting time of blood has been achieved.[7]

The following studies were carried out to determine whether Polybrene might be similar to either protamine or heparin in its anticoagulant action.

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†Polybrene powder (Serial 717-7725), Abbott Laboratories, North Chicago, Ill.

Materials and methods

Venous blood was obtained from normal young adults by a 2-syringe technique using siliconized syringes and 18 gauge needles.

Whole blood clotting time. A modified 3-tube Lee-White method was used at 37° C. One milliliter of blood was put into each tube (10 by 75 mm.) with a glass bead at the bottom. The tubes were tilted every 30 seconds. The clotting time recorded was that of the third tube.

Whole blood and plasma recalcification clotting times. The clotting time of 0.2 ml. of citrated or oxalated blood or plasma, to which was added 0.2 ml. of 0.02 M $CaCl_2$, was determined at 37° C.

Prothrombin time. This was determined by the method of Quick,[8] using a liquid rabbit brain thromboplastin* and 0.02 M $CaCl_2$.

Prothrombin consumption time, serum prothrombin time, and thrombin time. These were determined according to methods of Quick.[8]

Thromboplastin generation test. Performance of this test was as previously described.[9] Unless otherwise indicated, "cephalin," prepared according to the method of Bell and Alton,[10] was used instead of platelets.

Polybrene. Because of its hygroscopic properties, the Polybrene powder was stored in a desiccator over $CaCl_2$ pellets. All solutions were made in 0.85 per cent saline.

Thrombin. Thrombin, topical† (5,000 National Institutes of Health units per 5 ml.) was used. All dilutions were made in distilled water.

Fibrinogen. Bovine fibrinogen,‡ fraction I from bovine plasma, was used. All solutions were made in phosphate buffer, pH 7.45.

Table I. Effect of Polybrene on the whole blood clotting time (WBCT) and prothrombin consumption time (PCT) of whole blood.

	1		*2*		*3*	
Polybrene (gammas in 0.01 ml.)	*WBCT (1.0 ml.) (minutes)*	*PCT (seconds)*	*WBCT (1.0 ml.) (minutes)*	*PCT (seconds)*	*WBCT (1.0 ml.) (minutes)*	*PCT (seconds)*
0	9	45½	7½	26½	10½	24½
25	10½	31	7½	18		
50	12	27			13½	23
55	12½	24	9½	16½		
60	13½	25	11½	14	15½	24½
65	16	13½	11½	14		
70	23½	13	12	13½	18	13
75	33	13	12	11½		
80					19½	13½
90					25½	13

Results

Whole blood clotting time and prothrombin consumption time (Table I). When whole blood was added to increments of Polybrene in glass tubes at 37° C., the clotting time was progressively prolonged. At the same time, prothrombin utilization was decreased. In some instances, as can be seen in Table

*Permaplastin, C. W. Alban & Co., St. Louis, Mo.
†Parke Davis & Co., Detroit, Mich.
‡Armour Laboratories, Division of Armour & Co., Kankakee, Ill.

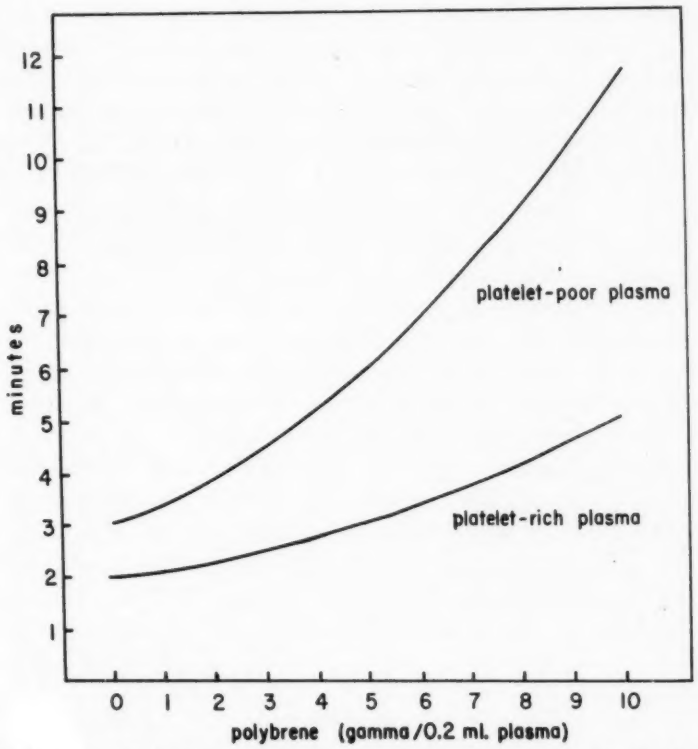

Fig. 1. The effect of Polybrene on the recalcification clotting time of platelet-rich and -poor plasma.

I, lower doses of Polybrene lengthened the clotting time without appreciably affecting prothrombin consumption. Comparable results were obtained with the effect of Polybrene on the recalcification clotting time of citrated blood. It was also noted that with the higher dosages of Polybrene (80 and 90 gammas per milliliter), marked agglutination of erythrocytes occurred. In addition, erythrocyte sedimentation was enhanced in the presence of Polybrene and at doses lower than those causing erythrocyte agglutination.

Plasma recalcification clotting time. Increments of Polybrene also progressively prolonged the recalcification clotting time of plasma. Identical results were obtained whether the plasma was derived from oxalated or citrated blood. However, the degree of clot inhibition by Polybrene was found to be dependent upon the number of platelets in the plasma. In Fig. 1, one sees the difference between the effect of Polybrene on platelet-rich plasma and -poor plasma. The platelet-rich plasma, obtained by centrifuging blood at 800 r.p.m. for 5 minutes, contained about 400,000 platelets per cubic millimeter. The platelet-poor plasma was obtained from blood centrifuged at 3,000 r.p.m. for 10 minutes. The critical level of platelets, below which significant sensitivity to Polybrene could be demonstrated, was found to be about 100,000 per cubic millimeter.

Thromboplastin generation test. Polybrene, added to a thromboplastin generation mixture, inhibits the formation of "thromboplastic" activity, or, if added to the mixture after the point at which optimum activity is reached, causes deterioration of activity. The effect of various concentrations of Polybrene on the thromboplastin generation test is seen in Fig. 2. The degree of inhibition by Polybrene is reproducible, but varies among blood samples from different individuals, a fact similar to that found in inhibition by heparin.[11] However, at least 1 gamma per milliliter of generation mixture must be present to produce significant inhibition of "thromboplastic" activity production.

Inhibition of thromboplastin generation is not due to carry-over of Polybrene from the generation mixture to the one-stage prothrombin time determination phase of the procedure, inasmuch as the addition of amounts up to 5 gammas to each substrate tube of a normal thromboplastin generation test in no way changed the curve of activity production.

The final degree of activity appears to be constant for a fixed amount of Polybrene regardless of at which stage of generation the Polybrene is added to the mixture. In Fig. 3 are the thromboplastin generation curves obtained when 3 gammas of Polybrene were added to the generation mixture before the onset of activity production, i.e., before calcification, during the lag phase of activity development, at the point of optimum activity, and after this point.

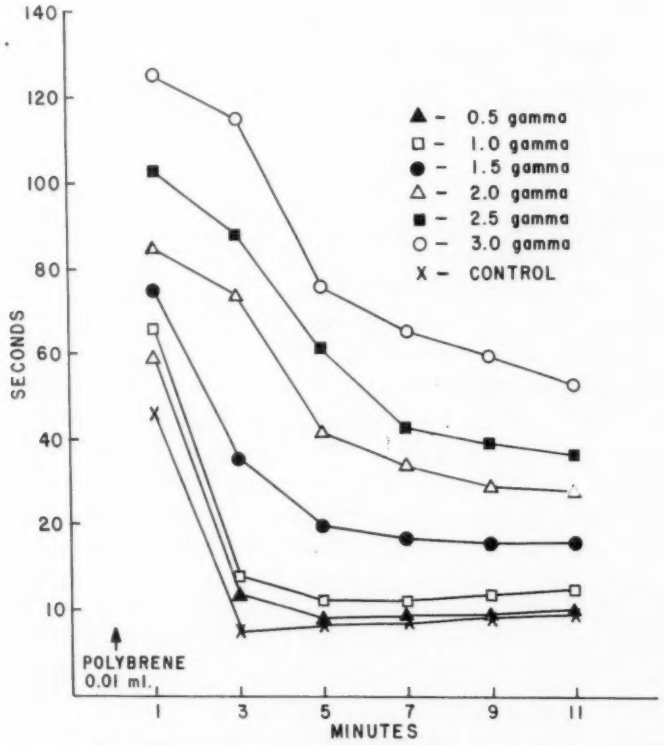

Fig. 2. The effect of Polybrene on the thromboplastin generation test. The Polybrene was added to the mixture prior to the addition of cephalin and calcium.

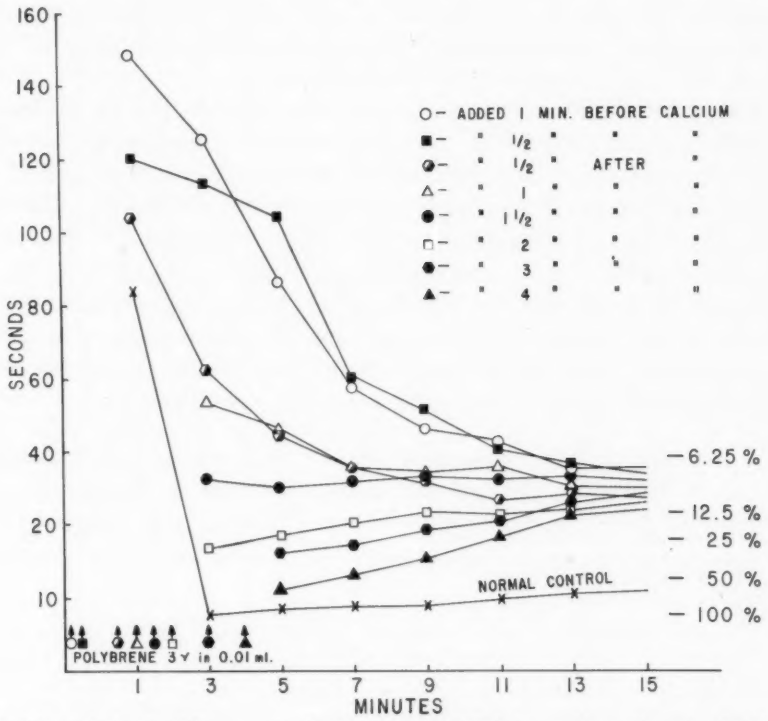

Fig. 3. The effect of adding a constant amount of Polybrene to a thromboplastin generation test at various stages of generation.

As can be seen, Polybrene either inhibits the production or, if already formed, causes partial deterioration of activity. In any event, a constant degree of activity finally is reached which is relative to the amount of Polybrene added.

As with inhibition of thromboplastin generation by protamine[9] as well as heparin,[12] increasing the concentration of the "cephalin" or of the adsorbed plasma had no effect on inhibition by Polybrene. However, if a platelet suspension was substituted for the "cephalin," twice as much Polybrene was necessary to bring about an equivalent degree of inhibition. This corresponds to the results obtained with the recalcification clotting time of platelet-poor and -rich plasma. Moreover, increase of the concentration of platelets in the mixture tended to overcome inhibition by Polybrene.

In addition, as in the case of heparin[12] or protamine,[9] inhibition of thromboplastin generation by Polybrene could be counteracted by increasing the concentration of the serum component (using a serum dilution of 1:2 or less rather than 1:10).

If normal serum were diluted 1:10 in $BaSO_4$-adsorbed serum instead of saline, equivalent doses of Polybrene had less effect on thromboplastin generation. This was not due to the presence of factor V (labile factor, proaccelerin). Aged adsorbed serum was as effective as fresh adsorbed serum, and neither one had any appreciable factor V activity as compared to fresh adsorbed plasma which does, and which, in increased concentrations, did not correct inhibition by Polybrene.

To determine whether the correction of inhibition with Polybrene by increased concentrations of serum were due to platelet-like activity of serum (PLAS),[13] the following procedure was carried out.

Blood was secured with siliconized equipment and chilled in an ice bath. It was then spun in a refrigerated centrifuge at 4° C., first at 800 r.p.m. for 5 minutes to obtain platelet-rich plasma and then at 3,000 r.p.m. for 15 minutes to obtain platelet-poor plasma. The plasmas were transferred to glass tubes either empty or containing Permaplastin (0.1 ml. Permaplastin per milliliter of plasma) and placed in a 37° C. water bath for at least 2 hours. The sera obtained from these plasmas were used in the thromboplastin generation test and compared with serum obtained from whole blood clotted with and without Permaplastin (0.25 ml. per 5 ml. blood). Serum prothrombin times were determined on all specimens prior to use. These are listed in Table II.

Table II. Prothrombin time determinations on sera used in thromboplastin generation test to determine effect of PLAS on inhibition by Polybrene

Derivation of serum	*Serum prothrombin time (seconds)*
Whole blood	25
Whole blood + Permaplastin	> 180
Platelet-rich plasma	64
Platelet-rich plasma + Permaplastin	> 180
Platelet-poor plasma	13
Platelet-poor plasma + Permaplastin	> 180

All sera gave a normal curve of thromboplastin generation except for the one derived from platelet-poor plasma, in which case the lag phase was eliminated by the high concentration of serum prothrombin. With the 1:10 dilution of serum no significant difference was noted in the degree of inhibition by a fixed amount of Polybrene. However, in the mixture containing the serum from platelet-poor plasma, the rate of production of activity was faster. When the serum concentration was increased to full strength, although in all cases normal "thromboplastic" activity was reached in the presence of Polybrene, the rate of generation varied. Listed in the order of decreasing rates they were as follows: serum derived from platelet-poor plasma; whole blood; platelet-rich plasma; platelet-rich plasma plus Permaplastin; whole blood plus Permaplastin; and platelet-poor plasma plus Permaplastin. It can be seen that the greater the potential of thrombin generation in the mixture, the less the Polybrene inhibits the production of "thromboplastic" activity. When the prothrombin content of the serum is eliminated, it would appear that the PLAS does play a role in counteracting inhibition of activity by Polybrene in the thromboplastin generation test.

As also was found with protamine,[9] the extent of inhibition of the thromboplastin generation test by Polybrene varied inversely with the degree of factor IX activity (plasma thromboplastin component, Christmas factor). The abnormality of the thromboplastin generation test on an individual with a congenital factor IX deficiency was increased by the addition of Polybrene to the

mixture. If a small amount of normal serum was added to the factor IX deficient serum (1:4), the abnormal thromboplastin generation test was corrected, and this mixture was less sensitive to Polybrene inhibition. If the concentration of factor IX deficient serum alone was increased to full strength, the degree of inhibition by Polybrene was the same as for the 1:10 dilution of factor IX deficient serum corrected by normal serum. Part of this decreased resistance to inhibition of Polybrene by the full strength serum could be due to the higher prothrombin content of the serum, ergo, to increased thrombin generation.

The inhibition of the thromboplastin generation test by Polybrene could also be counteracted with heparin. When the two were combined before addition to a generation mixture the ratio of "neutralization" was 1:1. If the Polybrene was added to the mixture first and the heparin immediately thereafter, half as much heparin was required to yield or restore normal "thromboplastic" activity. This change in the correction ratio is the same as that reported for heparin correction of inhibition by protamine[9] and for protamine correction of inhibition by heparin.[14] The theory previously offered for the change in the correction ratios, namely, that of 2 different reactive sites, appears to be invalid, since in the case of Polybrene its reacting sites are of only one type.

Table III. Effect of Polybrene on the one-stage prothrombin time of oxalated plasma

Polybrene (gammas)	*Prothrombin time (seconds)*	
0	12.2	Solid clots
2.5	12.2	
5	12.5	
10	13.7	
25	17	Stringy clots
50	20	
75	23	
100	25	
150	27	
200	27	

One-stage prothrombin time. Increments of Polybrene added to the plasma in the one-stage prothrombin time determination brought about prolongation of the clotting time as well as a decrease in the quality of the fibrin clot formed (Table III). No difference in the results was observed if plasma from oxalated or citrated blood was used, or if the Polybrene was incubated for periods of time with either the plasma or the thromboplastin. Increasing the concentration of Polybrene to as high as 4,000 gammas per milliliter of plasma did not decrease the prothrombin time to a lower level than that of 20 per cent activity. Polybrene, in higher concentrations, similarly inhibited the one-stage prothrombin time determination when "blood thromboplastin" was used in place of tissue thromboplastin.

Thrombin clotting time of plasma and fibrinogen. As opposed to the inhibition of the tests of the first and second stages of blood coagulation, Polybrene

enhanced the thrombin clotting of both plasma (Table IV) and solutions of fibrinogen (Table V).

In contrast to the effect of protamine on the thrombin-fibrinogen system,[15] high concentrations of Polybrene accelerate the reaction as much as lower concentrations. When added to plasma, no appreciable precipitation of fibrinogen could be determined. However, when added to a solution of fibrinogen, increments of Polybrene produced an increasing amount of precipitate. The removal of this precipitate did not alter the coagulability of the substrate by thrombin. The precipitate was soluble in 3 per cent saline and the solution could be clotted with thrombin, the amount of clot being relative to the volume of the original precipitate.

Table IV. Effect of Polybrene on thrombin clotting time of oxalated plasma

*Polybrene (gammas in 0.01 ml.) added to 0.2 ml. plasma**	+	*Thrombin 0.1 ml. (seconds)*	
0		7	8
2.5		7	8
5		7	8
10		6½	7
25		6½	7
50		6	7
100		6	6
200		6	6
400	(0.02 ml.)	6	6
800	(0.04 ml.)		6
2,000	(0.1 ml.)		7
Saline	0.1 ml.		10.2

*No visible precipitation of fibrinogen occurred in any of the tubes.

Table V. Effect of Polybrene on the thrombin clotting time of fibrinogen

Polybrene (gammas in 0.01 ml.) added to 0.2 ml. fibrinogen	+	*Thrombin 0.1 ml. (seconds)*
0		10 (9)*
2.5		7 (7)
5		6 (6)
10		5 (5)
25		5 (5)
50		5 (5)
75		5 (5)
100		5 (5)
150		5 (5)
200		5 (5)

*The figures in parentheses represent the clotting time of the supernatant after removal of the precipitated fibrinogen by centrifugation.

Discussion

In the present study, Polybrene was indeed found to delay whole blood clotting in vitro and in doses similar to those found for dog blood.[5] Simultaneously, it was noted that decreased prothrombin consumption occurred in

bloods in which clotting was retarded with Polybrene. This was corroborated by the finding that Polybrene inhibited the production of optimum clot-promoting activity in the thromboplastin generation test. It was also discovered that the number of platelets present influenced the degree of retardation by Polybrene of the clotting of recalcified plasma as well as the generation of "thromboplastin." Inhibition of the thromboplastin generation test by Polybrene did not seem to be due to interference with factor V, factor VIII, or platelet factor 3 activity. Increasing the concentration of these factors in the generation mixture did not affect inhibition by Polybrene as did an increased concentration of platelet material. An increased concentration of the serum component in the thromboplastin generation test mixture also counteracted the inhibition of activity. This would appear to be due to various factors present in serum, namely, prothrombin, factor IX, and PLAS. In addition, one must consider the possible influence of complexes formed between Polybrene and various serum proteins not concerned in the coagulation mechanism.

Polybrene also prolonged the conversion of prothrombin by tissue thromboplastin. However, much larger concentrations of Polybrene were required than those needed to interfere with tests concerning the first stage of coagulation. No appreciable difference was found if Polybrene was incubated with the plasma or the thromboplastin before performance of the test.

In contrast to its inhibitory action in the preceding tests, Polybrene accelerated the conversion of fibrinogen to fibrin by thrombin. Polybrene did precipitate some of the partially purified fibrinogen, but not enough to interfere with the thrombin-fibrinogen reaction. It did not, however, precipitate any appreciable amount of fibrinogen from plasma.

Protamine, too, has been shown to retard blood coagulation in vitro,[16-20] to be inhibitory in the thromboplastin generation test,[21] particularly by interfering with factor IX activity,[9] to impede prothrombin conversion by both tissue thromboplastin[18, 19, 21, 22] and "blood thromboplastin,"[21] to be fibrinoplastic in the thrombin-fibrinogen reaction,[15, 18, 23] and to cause agglutination of erythrocytes.[16, 24] Inhibition by protamine in the thromboplastin generation test can also be counteracted by heparin.[9]

In contrast to Polybrene, protamine effectively precipitates fibrinogen,[18, 24, 25] and has the same influence on the thromboplastin generation test whether platelet suspension or "cephalin" is used in the mixture.[9]

Unlike heparin, Polybrene is not antithrombic. It is of interest, however, that as with heparin,[26, 27] thrombocytopenic blood or plasma is more sensitive to the anticoagulant action of Polybrene. Whether the antiheparin fraction of platelets (platelet factor 4),[28-32] is also anti-Polybrene remains for further investigation. Polybrene, as does heparin, has an antagonism toward factor IX activity[12, 33, 34] and the PLAS.[33]

No reports of the effect of Polybrene on thrombocytopenic blood in vivo have been published. However, a lowering of the platelet count has been observed in individuals who received an intravenous injection of Polybrene.[6] Since, during the use of extracorporeal blood oxygenators, a decrease in platelets is often observed,[35] one wonders whether Polybrene might adversely affect

reconstitution of the coagulability of heparinized blood in such cases. In reporting the use of their own blood oxygenator, Panico and Neptune[36] stated, "After the catheters are removed, the patient receives adequate Polybrene and *two units of platelets* intravenously to enhance rapid blood coagulation."*

Therefore, although the concentrations of Polybrene required to influence normal blood coagulation in vitro are somewhat higher than those usually recommended in its clinical use as an antiheparin,[6] its margin of safety as far as overdosage is concerned may be much less than that of protamine if the platelet count is lowered.

We have shown that Polybrene affects blood coagulation in various ways. How it affects it is difficult to explain. Polybrene is a polyelectrolyte, as are heparin and protamine. As such, it may act by either emulsification or neutralization of molecules. A clearer understanding of exactly how these substances exert their action on blood may be the clue to the mechanism of blood coagulation itself.

The authors wish to express their appreciation to Dr. Norman C. Wheeler, Abbott Laboratories, for the supply of Polybrene powder used in this study.

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Impairment of hemostasis in the urinary tract: The role of urokinase

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Urokinase, an activator of the plasminogen-plasmin system, is normally present in urine, and confers upon it the ability to lyse fibrin clots. Though the source of urokinase is still an unsettled question, it has been suggested that urokinase may play a role in maintaining the patency of the urinary tract by lysing fibrinous deposits. Since such an action might also serve to impair hemostasis in the urinary tract, an investigation was undertaken of the effect of inhibiting urinary urokinase activity on the duration and extent of the bleeding observed following prostatectomy. Such a study was made possible by the availability of ε-aminocaproic acid, a potent inhibitor of urokinase activity, which, when administered intravenously in appropriate dosage, was promptly excreted into the urine in concentrations sufficient to inhibit urokinase activity. It was found that, in association with the inhibition of urokinase activity by this acid, there was a significant (fourfold) reduction in blood loss in ε-aminocaproic acid treated patients as compared to the reduction obtained with control subjects. Levels of this acid in the urine were measured, and the excretion rates and relationship between its concentration and urokinase inhibition are discussed. The experimental results provide evidence to support the concept that urokinase can impair hemostasis in the urinary tract and that its inhibition may be attended with improved hemostasis.

It has been known for many years that urine can digest impure fibrin clots,[1] an effect now attributed to the presence in urine of a plasminogen activator named urokinase.[2-4] Plasminogen, a plasma globulin which occurs naturally in close association with fibrin, is converted by urokinase to plasmin,

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a proteolytic enzyme: clot lysis ensues. Other activators of the plasminogen-plasmin system include plasma activator, tissue activators, and bacterial activators, e.g., streptokinase.

Urokinase, itself a proteolytic enzyme, activates plasminogen by first order kinetics, probably by splitting arginine and/or lysine bonds.[5, 6] It is a colorless protein with a high degree of stability over a wide pH range.[7]

Smyrniotis and associates[8] have shown that urokinase excretion rates in normal human subjects are independent of age, sex, and urine volume, and Bjerrehuus[9] observed that urokinase concentrations were similar in bladder urine and urine obtained from the renal pelvis. The work of von Kaulla and Swann,[10] and of Smyrniotis and associates[8] suggests that urokinase may represent, at least in part, excreted plasma plasminogen activator. The current state of knowledge, relative to urokinase, has been the subject of a recent review article.[11]

The experiments described in the present communication were designed to study the possibility that urokinase, by activating clot plasminogen, might result in impairment of hemostasis in the human urinary tract.

The problem was approached by the use of ϵ-aminocaproic acid (EACA), a synthetic aminocaproic acid, which has been shown,[12, 13] at concentrations as low as 10^{-4} molar, to be a potent competitive inhibitor of plasminogen activation by urokinase and streptokinase. EACA was originally thought to act as a plasmin inhibitor,[14] and, though in high concentrations, e.g., 10^{-2} molar or above, it does inhibit plasmin to some extent, in lower concentrations its action is primarily that of an activator inhibitor. Sherry and co-workers[15] demonstrated in man that the intravenous infusion of EACA inhibited the plasma plasminogen activator induced by the systemic administration of streptokinase, bacterial pyrogen, and nicotinic acid.

The clinical investigative model used in the present work was the patient after transurethral prostatectomy. Such patients are excellent for study because of the relative uniformity of the surgical trauma and the ease of obtaining complete urine collections.

After prostatectomy, urine containing urokinase flows over the clot in the raw prostatic bed: it is postulated that clot plasminogen is activated to plasmin and clot lysis and impaired hemostasis ensue. Were such a mechanism operative, the administration of EACA to patients after prostatectomy, in sufficient quantity to inhibit markedly urinary urokinase activity, should be accompanied by reduced blood loss, thus providing support for the concept that urokinase may, in some circumstances, cause impairment of hemostasis in the urinary tract. The experimental results to be reported were entirely consistent with the hypothesis cited; the administration of EACA resulted in a significant (fourfold) reduction in blood loss as compared to results obtained in the control group.

A small group of patients was also studied after suprapubic prostatectomy, and advantage was taken of the administration of EACA to study its metabolism and to relate changes in urokinase activity to EACA levels.

Materials and methods

Urinary blood content. This was measured as hemoglobin by a modified cyanmethemoglobin method. To 9 ml. of urine was added 1 ml. concentrated Drabkin's solution.* The specimen was centrifuged at 3,000 r.p.m. for 10 minutes; if a deposit of unhemolysed red cells was present, it was dissolved in 0.2 ml. of 30 per cent urea and added to the supernatant. Optical density was read at 540 mμ in a Beckman DU spectrophotometer,† using 9 ml. pooled normal urine with 1 ml. Drabkin's solution as a blank. Urine samples were diluted with water as necessary to keep optical density readings below 0.900. Hemoglobin content in grams was obtained by reference to a standard curve. The method is sensitive and reproducible; the lower limit of detection of hemoglobin is about 0.03 mg. per milliliter (equivalent to 0.2 ml. of normal blood per liter of urine). EACA added to urine in concentrations up to 10^{-1} molar did not affect optical density readings in the hemoglobin assay.

Urinary urokinase assays. The assays were performed by the method of Smyrniotis and associates,[8] utilizing the unheated fibrin plate technique of Mullertz.[16] Plates were prepared as described by Alkjaersig and associates.[12] With this method, assay of the reference urokinase solution used throughout the study resulted in a standard deviation of approximately 11 per cent. Results are expressed in units which are closely comparable with those of Smyrniotis and co-workers,[8] and are about one-eighth the potency of the Ploug unit.[7]

In order to study the effects of EACA on urokinase excretion rates, as well as the inhibitory action on urokinase activity, urine specimens were assayed both before and after dialysis. Dialysis was carried out for 8 hours at 4° C. against a large volume of constantly stirred 0.01 molar phosphate buffer, pH 7.6, and always was associated with loss of urokinase activity (mean of 35 per cent throughout the investigation). This loss of activity was also seen when the standard urokinase was dialysed. The loss of activity may be due to coprecipitation of urokinase with mucoprotein: a contributing factor is the slight increase in volume (about 5 per cent), which usually was associated with dialysis.

Hemoglobin or aged whole blood did not affect the urokinase assay; standard urokinase diluted with saline, when compared with standard urokinase diluted with the same volume of bank blood, gave identical results. It is possible that fresh blood containing high levels of plasma activator, if mixed with urine, could result in higher levels of urinary activity, but on a quantitative basis (see below), this factor would seem to have been of negligible importance in the present investigation.

Urinary EACA estimations. These were carried out by high voltage paper electrophoresis, using a slightly modified version of the technique described by Sjoerdsma and Hanson.[17] An E-C horizontal high voltage apparatus‡ was used, with Schleicher and Schuell paper No. 598. Samples were run for one hour at 1,000 v. After staining by the ninhydrin-copper method, EACA bands, located by reference to a standard run with each assay, were eluted and read at 508 mμ in a Beckman DU spectrophotometer. The molar extinction coefficient of EACA, treated in this manner, was 0.35.

Euglobulin lysis times. These were expressed in units of fibrinolytic activity.[18]

Transurethral prostatectomy patients. Twenty-seven patients, whose ages ranged from 41 to 79 years (mean, 68 years), were studied. Twelve patients, selected at random, were treated with EACA; 15 were control subjects. The EACA-treated and the control series were comparable with regard to preoperative hemoglobin levels (mean, 13.3 ± 1.9 Gm. and 13.5 ± 1.8 Gm., respectively), and preoperative blood urea nitrogen levels (17.8 ± 4.6 mg. per 100 ml. and 19.4 ± 6.7 mg. per 100 ml., respectively).

Transurethral electro-resections (McCarthy resectoscope) were carried out by the Urological Service. In all patients, bladder washouts were free from macroscopic blood before the patients left the operating room, and it should be emphasized that only bleeding which began postoperatively was studied. EACA infusions (see below) were commenced within 10 minutes of the end of operation. In the EACA-treated group, histologic examination of

*Reagent obtained from Fisher Scientific Co., Pittsburgh, Pa.
†Beckman Instruments, Inc., Palo Alto, Calif.
‡E-C Apparatus Co., Swarthmore, Pa.

the resected tissue showed 7 cases with benign nodular hyperplasia, one (5 Gm. of tissue resected) with fibrosis only, one with fibrosis and chronic inflammation, one with leukemic infiltration, and one with well-differentiated adenocarcinoma of the prostate. In the control group, 12 had benign nodular hyperplasia and fibrosis, and 2 had well-differentiated adenocarcinoma of the prostate. Mean weight of tissue resected was, in the treated group, 14.6 ± 9.2 Gm., and in the control group, 15.4 ± 7.4 Gm.

Suprapubic prostatectomy series. Fifteen patients, whose ages ranged from 57 to 82 years (mean, 71 years), were studied. Seven patients selected at random were treated with EACA, and 8 were control subjects. Mean preoperative hemoglobin levels were 14.6 ± 2.6 Gm. per 100 ml. for the EACA-treated patients, and 14.6 ± 2.2 Gm. per 100 ml. for the control subjects. Mean blood urea nitrogen levels were 18.0 ± 10.7 mg. per 100 ml. for the EACA-treated patients, and 16.8 ± 2.2 mg. per 100 ml. for the control group. Histologic examination of the resected tissue showed benign nodular hyperplasia in each case. No evidence of carcinoma was found in any specimen. Mean weight of tissue removed was 48.2 ± 38.3 Gm. in the EACA-treated group and 72.0 ± 40.4 Gm. in the control group. This difference in the amount of tissue removed, inevitable in a small series selected at random, was not significant ($p > 0.2$).

BLOOD LOSS AFTER OPERATION.* This was measured by collection of all drainage (per urethram and suprapubic) and by elution of dressings.

EPSILON AMINOCAPROIC ACID.† EACA was obtained as a sterile 10 per cent aqueous solution. In all patients, the EACA was administered intravenously at a rate of 1 Gm. per hour for 4 hours, then at 0.5 Gm. per hour for 8 hours (total dose, 8 Gm.). In 7 patients, the EACA was added to the routine postoperative intravenous infusion (usually about 1,500 ml. over the 12 hour period) and in 5 patients the EACA was administered using a constant rate infusion pump.‡

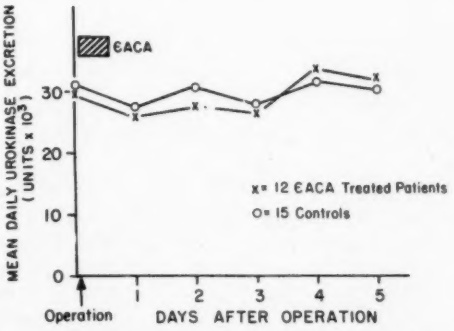

Fig. 1. Data on urokinase excretion in the control and EACA-treated groups. Assays were conducted after dialysis of urinary specimens (see *Methods*). The results indicate that EACA administration does not alter urokinase excretion rates.

Results

Transurethral prostatectomy series.

UROKINASE ACTIVITY. This activity was followed for 5 days after operation. Assays also were made before operation, and each sample was assayed after dialysis to permit comparison of true urokinase excretion rates between treated and control groups.

*Intravascular hemolysis as a cause for hemoglobinuria was excluded by inspection of postoperative plasma specimens.

†Kindly supplied by Dr. Gilbert Bayne of Merck Sharp & Dohme Research Laboratories, West Point, Pa.

‡Harvard Apparatus Co., Dover, Mass.

Fig. 1 demonstrates that EACA administration did not alter true urokinase excretion rates as urokinase assays on dialysed specimens from the 2 subject groups did not differ significantly ($p > 0.1$). Similarly, it is apparent that in neither group did urokinase excretion alter as a result of the operative procedure.

Fig. 2 illustrates urokinase assays performed on undialysed urine specimens obtained from the two subject groups. It will be seen that, in the control subjects, urokinase activity continued unchanged following operation. In contrast, in the EACA-treated group, assayable urokinase activity, which was similar preoperatively to that of the control group ($p > 0.1$), was zero on the first postoperative day, and a mean of 28 per cent of preoperative levels on the second day ($p < 0.01$). Even by the third day, activity in the EACA-treated group had not fully returned to preoperative levels though the depression is not significant at the 5 per cent level.

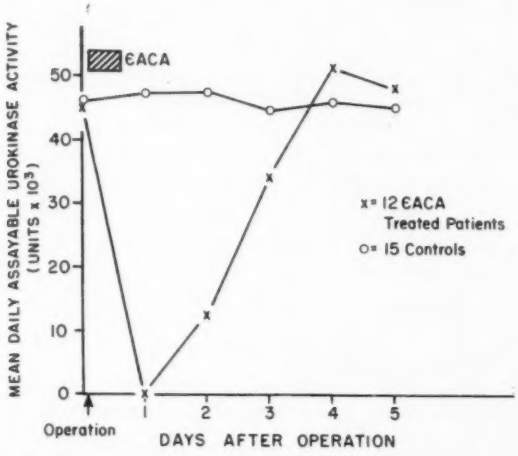

Fig. 2. Effect of EACA treatment on average daily assayable urokinase in control and treated groups. EACA administration caused profound inhibition of assayable urokinase activity, though, as shown in Fig. 1, it did not alter urokinase excretion.

The data displayed in Figs. 1 and 2 indicate that the effect of EACA administration was to inhibit the biochemical action of urokinase as a plasminogen activator. There was no evidence to suggest that EACA administration either altered urokinase excretion or destroyed the material.

BLOOD LOSS. The blood loss was studied for 5 days following operation (in one treated patient and 2 control subjects, for 4 days only). As shown in Fig. 3, blood loss of the EACA-treated patients was strikingly reduced as compared to that of the control group. As might be expected, the difference was most marked on the first postoperative day, when there was a fourfold reduction in blood loss in the EACA-treated patients, as compared with that in the control group. The range of blood loss over the 5 day period in the EACA-treated group was from 0.5 to 5.2 Gm. hemoglobin; in the control group, from 2.5 to 45.6 Gm. hemoglobin.

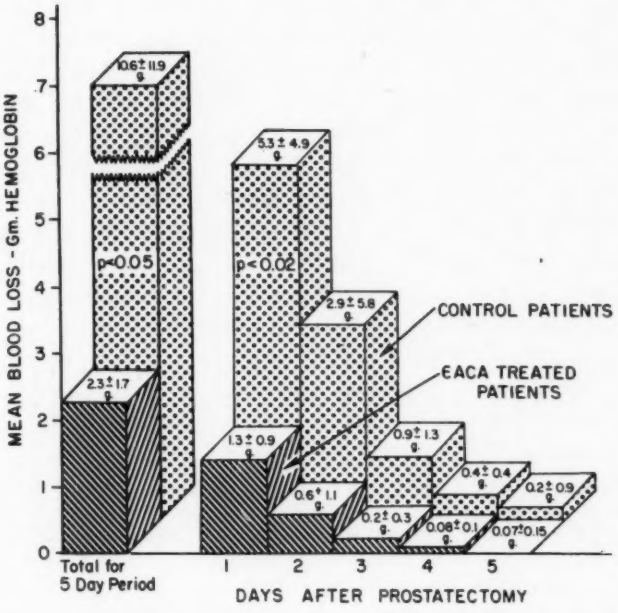

Fig. 3. Daily and total (5 day period) blood loss after transurethral prostatectomy. The mean results for 12 patients treated with EACA are compared with the results obtained in 15 control subjects. EACA treatment caused a marked reduction in postoperative hematuria, particularly on the first and second postoperative days.

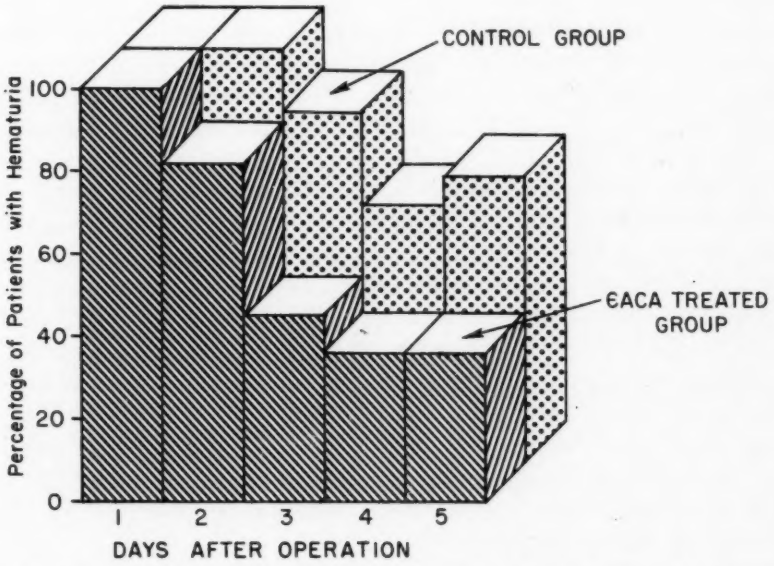

Fig. 4. Effect of EACA administration on the duration of urinary bleeding following trans urethral prostatectomy. Shown are the percentage of patients in the control and treated groups with blood in the urine on successive days after surgery. Significantly fewer specimens contained blood in the treated group than in the control.

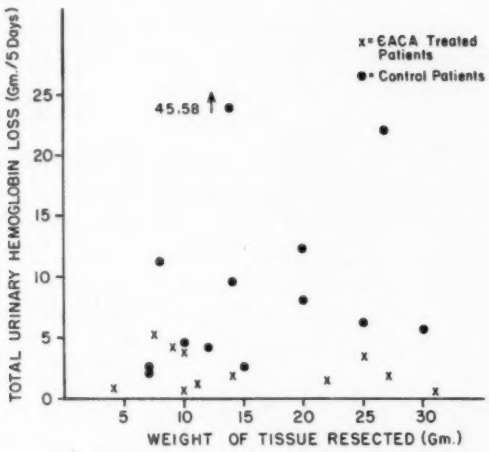

Fig. 5. Lack of relationship between total urinary hemoglobin loss (5 day period) and weight of prostatic tissue resected. The effect of EACA treatment can be seen in the much smaller range of blood loss in the treated patients as compared with results obtained in the control subjects.

Table I. Effect of EACA on urinary blood loss in patients after suprapubic prostatectomy (mean values and standard deviations are shown)

	Hemoglobin loss following surgery (Gm. per day)					*Total loss (5 day period)*
Subjects	*1*	*2*	*3*	*4*	*5*	*Gm. hemoglobin*
7 EACA-treated patients	14.7 ± 11.9	5.0 ± 2.3	3.5 ± 1.6	2.4 ± 1.4	1.4 ± 0.6	26.9 ± 14.9
8 control subjects	41.3 ± 27.8	8.9 ± 6.9	3.5 ± 1.7	2.4 ± 1.3	1.3 ± 0.9	57.5 ± 33.3
p values	< 0.05	< 0.1				< 0.05

The duration of bleeding in the EACA-treated patients was also reduced, as is shown in Fig. 4, which is a comparison of the percentage of patients observed for the full 5 day period (in both treated and control groups) who had measurable blood loss on successive postoperative days. For the 11 EACA-treated patients followed for the full 5 day period, there was blood in 33 daily samples, but no bleeding was detected in 22 samples; for the 13 control subjects, there was blood in 54 samples, and only in 11 was no blood detected. The difference between the two groups was significant ($\chi^2 = 7.957$, $p < 0.02$).

As was anticipated, no correlation was observed between preoperative urokinase levels and blood loss following surgery. Nor, as shown in Fig. 5, did a significant relationship exist, in this series, between postoperative bleeding and the amount of prostatic tissue resected.

Suprapubic prostatectomy series. Blood loss for the EACA-treated and the control patients is described in Table I. While the changes were somewhat less striking than those observed in the transurethral series, a threefold reduction of postoperative hematuria was recorded on the first day of treatment ($p < 0.05$).

URINARY EACA ESTIMATIONS. These were carried out on samples from 7 patients for 4 days after operation. Results are shown in Table II, which records the mean daily EACA concentration, the total daily excretion of EACA, the per cent of the administered dose eliminated on each day, and the per cent of the urokinase activity inhibited. It will be noted that approximately 60 per cent of the administered dose was eliminated during the first day; this resulted in urinary EACA concentrations of above 10^{-2} molar and complete inhibition of urokinase activity. In 4 patients the urinary EACA excretion was measured for the 12 hour infusion period and for the succeeding 12 hour postinfusion period. A mean value of 38.2 ± 6.8 per cent (3.1 ± 0.5 Gm.) of the administered dose was excreted during the infusion period, and a mean value of 18.8 ± 1.2 per cent (1.5 ± 0.1 Gm.) of the dose was excreted in the next 12 hours. During the second day, approximately 20 per cent of the administered dose was found in the urine; the urine concentrations were in the range of 6×10^{-3} molar and approximately 60 per cent of the urokinase activity was inhibited. By the third day, EACA was detected only in the urine from 2 subjects, in concentrations of 1.4×10^{-3} molar and 7.8×10^{-4} molar, respectively. In these 2 patients, there was a 16 and 13 per cent inhibition of urokinase activity. For the 7 patients, a mean value of 82.9 per cent of the EACA dose was recovered over the 4 day period of observation.

Euglobulin lysis times. In view of a possible increase in plasma fibrinolytic activity after prostatectomy, and the systemic fibrinolytic state that may complicate carcinoma of the prostate,[19] euglobulin lysis times were estimated preoperatively for the 3 patients with prostatic carcinoma, the patient with leukemic infiltration of the prostate, and 3 other patients. In each case, activity was less than 0.1 unit (within normal limits). In 5 control patients euglobulin lysis times were estimated with samples withdrawn 4 hours after the patients had entered the recovery room (usually about 10 minutes after the end of operation). The values were all less than 0.1 unit.

Table II. Daily urinary EACA excretion following administration of 8 Gm. EACA in 12 hour infusion to 7 patients following suprapubic prostatectomy (mean value and standard deviations are shown)

Observation	*Day*			
	1	*2*	*3*	*4*
EACA excreted (Gm./day)	4.8 ± 1.3	1.7 ± 0.98	0.1 ± 0.2	Nil
% of administered dose excreted	60.1 ± 16.0	21.3 ± 12.2	1.4 ± 3.0	Nil
Urinary EACA concentration (molarity)	$2.4 \pm 1.8 \times 10^{-2}$	$6.4 \pm 3.8 \times 10^{-3}$	$3.6 \pm 5.6 \times 10^{-4}$	Nil
% urokinase activity inhibited*	100	62.5 ± 5.0	8.0 ± 7.8	Nil

*Calculated on the basis of the predicted urokinase activity from preoperative observations. Levels of inhibition were similar when calculated from activity seen in dialysed specimens.

WHOLE BLOOD CLOTTING TIMES (LEE-WHITE). These were estimated in 4 EACA-treated patients in samples withdrawn the day before and 4 hours after operation. Normal values were obtained in all cases.

In view of the marked reduction in blood loss in EACA-treated patients as compared to that observed in control subjects, EACA was given to 4 patients not included in the controlled trial, in whom postoperative hematuria was a cause for concern. All patients showed an apparent response to therapy, but, because of the variable natural history of the disorder, the case history of only one is cited below.

Bleeding during suprapubic prostatectomy in an 80-year-old man was troublesome and persisted until EACA treatment was given 27 days later. In the 24 hours before EACA therapy, blood loss in the urine was estimated at 55.8 Gm. Eight grams of EACA was infused intravenously over 12 hours and 2 Gm. EACA was run into the bladder with a catheter. In the 24 hours during and following EACA infusion, blood loss was estimated at 0.3 Gm. Seven days later bleeding recurred, but was again promptly controlled with an EACA infusion. The striking response to EACA administration is displayed graphically in Fig. 6.

Toxicity. No evidence of thrombophlebitis was found following EACA infusion and there were no significant changes in body temperature, pulse rate, or blood pressure.

Brief case reports are given of 2 patients who presumably had vascular occlusion following EACA administration, though the occurrence of this accident in both instances was probably coincidental.

CASE 1. This patient was a 64-year-old man with a 17 year history of Parkinsonism and hypertension. There was a history of cerebral vascular occlusion 10 years previously. EACA was infused for 12 hours postoperatively. Three hours after the end of the EACA infusion the patient was found to be unresponsive and cyanotic. Consciousness returned in an hour or two when the patient had a left hemiparesis which persisted for several days. In a week, motor power was equal on both sides.

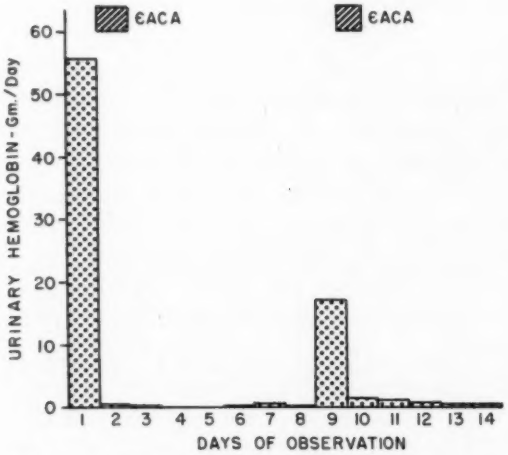

Fig. 6. The effect of EACA administration on the urinary hemoglobin loss in a patient with severe and protracted hematuria following surgery for prostatic adenocarcinoma. Prostatectomy was performed 27 days before EACA was first given.

CASE 2. A 74-year-old man, with a long history of ischemic heart disease and iatrogenic hypothyroidism, was in congestive heart failure at the time of surgery. EACA treatment was given for 12 hours postoperatively. His cardiovascular status improved somewhat during the postoperative period but, 10 days after operation and EACA treatment, he was found dead. Death was presumed to be due to vascular occlusion, cerebral, pulmonary, or coronary; no autopsy was performed.

Discussion

The presence in normal urine of substantial quantities of a plasminogen activator, urokinase, has posed problems concerning its source and possible physiologic role. Two views have been expressed: (1) urokinase represents an excretory product which reflects the dynamic state of the fibrinolytic enzyme system in vivo; and (2) urokinase is a secretory product of the renal epithelium, concerned primarily with maintaining the patency of the urinary tract by virtue of its ability to mediate the lysis of fibrin deposits. Some evidence exists to support both views, and it is not unlikely that the urokinase activity of the urine actually may represent the combined activity of several plasminogen activators derived from different sources.[11] Regardless of these differing views, the presence of urokinase in urine makes available an enzymatic mechanism for the lysis of fibrin deposits and blood clots in the urinary tract. It is recognized now that, under biological conditions, significant amounts of plasminogen, the precursor of the fibrinolytic enzyme, plasmin, are incorporated into the clot or fibrin deposit during its formation; the thrombi thus formed are endowed with an intrinsic enzyme system which, upon activation, results in the lysis of fibrin.[20] Under these circumstances, only the presence of a plasminogen activator in the surrounding fluid is required to mediate thrombolysis; indeed, there is evidence to suggest that activation of this intrinsic enzyme system of clots represents the major mechanism for thrombolysis under physiologic circumstances.[21]

The present study was undertaken to determine whether the presence of urokinase activity in the urine could contribute to the impaired hemostasis frequently observed in the urinary tract following ulcerating or traumatic lesions. Urine normally contains a thromboplastic material which, presumably, contributes to hemostasis in the urinary tract[22]; assays for this component were not performed because of the trauma of operation and the subsequent hematuria. The ability of the urine to lyse fresh fibrin deposits in the presence of operative trauma could play a significant role in maintaining continued bleeding, for it is well recognized that protracted hematuria, sometimes of a severe nature, may follow traumatic insults to the urinary tract. The availability of patients undergoing transurethral resection and suprapubic prostatectomy readily fulfilled the needs for clinical investigative models. The removal of prostatic tissue, which contains large amounts of a tissue plasminogen activator,[23] might, theoretically, remove a source of part of the urokinase activity, but, in view of the similarity of the assay values in control subjects in both the pre- and postoperative periods, such a contribution can only be considered as minimal. Furthermore, it is known that male and female urokinase output is approximately equal.[8] It was feasible to study the problem only by inhibition of urokinase activity, though

it would have been desirable to study also the effects of increased amounts of urokinase on hemostasis in the urinary tract. Inhibition of urokinase activity could readily be achieved by the systemic administration of EACA, since this powerful competitive inhibitor to plasminogen activation is poorly metabolized in vivo and rapidly excreted into the urine; in the rabbit this epsilon amino acid can be recovered virtually completely in the urine following its oral or systemic administration.[24]

In approaching the problem of EACA dosage and administration, two considerations seemed important: (1) to achieve a urinary EACA concentration sufficiently high to inhibit significantly urokinase activity; and (2) to sustain such concentrations for sufficiently long periods of time. From preliminary in vitro data, at the levels of urokinase activity present in normal urine, we could anticipate extensive inhibition with urinary EACA concentrations of 10^{-2} molar or above and moderate-to-extensive inhibition in the range of 10^{-3} to 10^{-2} molar. On the basis of preliminary data,[15] an 8 Gm. dose was infused intravenously over a 12 hour period. It was anticipated that, during this prolonged infusion, sufficiently large amounts of the amino acid would be excreted to achieve our initial objective, and, in addition, equilibration with the extravascular compartment would occur, followed by the slow elimination of the body pool of EACA in sufficient concentration to achieve a sustained inhibitory effect.

The data reveal that our objectives were achieved, though it is also likely that similar or even more desirable concentrations of urinary EACA could have been obtained by other modes of administration or different dosage schedules. The excretion rates observed in this study are much slower than those reported by Nilsson and associates,[25] who found that 40 to 60 per cent of a single oral or intravenous dose of EACA was recovered in the urine in 12 hours and only a small amount (2 to 4 per cent) was excreted in the next 12 hours; for more prolonged excretion of EACA, it would appear desirable to use multiple doses or sustained infusions. Further information concerning plasma levels of EACA, renal mechanisms for handling this substance, as well as other data concerning its distribution and metabolism, must await the development of an adequate plasma assay.

In both groups (transurethral resection and suprapubic prostatectomy) the data clearly indicate that the suppression of urinary urokinase activity was associated with a reduction in urinary hemoglobin loss. That the effect was more striking in the patients who underwent transurethral resections is not surprising, since, in the suprapubic prostatectomy procedure, much more extensive trauma is inflicted, direct visualization of bleeding points may be difficult, and reliance is placed on capsular retraction and packing to ensure hemostasis.[26, 27] Further, the weight of prostatic tissue removed is often, as in the present series, much larger in the suprapubic technique (mean, 61 Gm.) than in the transurethral procedure (mean, 15 Gm.). Nevertheless, the reduction was significant in both groups, and provides evidence that the urokinase activity of the urine contributed to the impaired hemostasis following the traumatic insult to the urinary

tract. Our present data do not entirely exclude the possibility that EACA administered by the intravenous route may have exerted a significant degree of systemic action on the plasminogen-plasmin system, or perhaps have exerted some hitherto undescribed action on hemostasis. However, it is emphasized that strong evidence has been adduced to support the hypothesis that EACA acted locally in the urinary tract. Conversely, the data also provide evidence to support the concept that urokinase, by promoting the lysis of fibrinous deposits, may play an important physiologic role in maintaining the patency of the urinary tract.

The present observations also bear on the broader subject of the relationship of the activity of the fibrinolytic enzyme system to the maintenance of normal hemostatic mechanisms and its contribution to certain hemorrhagic and thrombotic manifestations. It has become increasingly apparent that fibrinolysis, as a biological phenomenon, is in a state of dynamic activity; the factors regulating this activity and their contribution to disease are a matter of great current interest.[19, 28, 29] For this reason, the ability to inhibit the activity of plasminogen activators by EACA has obvious therapeutic implications. Recent studies indicate that this agent may prove to be extremely useful in the management of spontaneously occurring or induced systemic fibrinolytic disorders,[15, 25] and the present studies indicate a potential usefulness in the control of excessive bleeding arising under certain circumstances from the lower urinary tract.

Though no immediate or delayed local or systemic side effects were noted with the use of this agent in these patients, the possibility was entertained that EACA, by inhibiting normal plasma plasminogen activator activity, or the response to physiologic stresses, might predispose to the development of spontaneous thromboses. The 2 patients in our series with presumed vascular occlusion both had a background of vascular disease; in Case 2, death occurred 10 days later, and the diagnosis of vascular occlusion was not established. Further observations with EACA administration, concerning this potential danger, are indicated.

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Fibrinolysis V. The thrombolytic and anticoagulant activity of mold fibrinolysin (Aspergillin O) in vivo

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Detailed studies were carried out on the fibrinolytic and clot-delaying activity of Aspergillin O in 70 dogs. There was no evidence of toxicity or antigenicity with an average dose of 10 mg. per kilogram of weight and with repeated administrations at various intervals of time.

The effect of Aspergillin O in vivo lasted approximately one hour after the administration of the drug. There appeared fibrinolytic activity in whole blood and plasma without evidence of activation of profibrinolysin (plasminogen). There were also delayed formation and decreased yield of thrombin, moderate fibrinogenopenia, and some deficiency of clot accelerators. There was no significant acceleration of the clotting process with the dose employed. Venograms demonstrated the recanalization of venous vessels thrombosed for 24 to 48 hours, but Aspergillin O was unable to destroy thrombi older than 72 hours. The addition of heparin (0.2 mg. per kiloweight) enhanced the anticoagulant and fibrinolytic activity of the drug.

In dogs, Aspergillin O destroyed intravenous thrombi experimentally produced, while it delayed and interfered with the normal coagulation mechanism without hemostatic breakdown or acceleration of intravascular clotting. Its complex mechanism of activity suggested the potential use of the drug in the management of thromboembolic disease in man.

An agent totally effective in the management of arterial and venous thrombosis should arrest the continued intravascular deposition of fibrin,[1] and

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also induce recanalization of the vessel through total or partial destruction of the thrombus. The ability of a true anticoagulant agent such as heparin to prevent further propagation of the clot seems to have been proved. Yet, unless the thrombus is destroyed, irreversible vascular and tissular changes are bound to follow. Thus, prompt and sustained fibrinolysis in vivo represents a vital step in the ultimate management of throboembolism. Many agents have been tried to induce sustained fibrinolysis in vivo in man. A few have reached the stage of clinical trial. The use of streptokinase and of plasmin has given promising, although controversial, results.[2]

We have reported that an agent isolated from liquid cultures of Aspergillus oryzae B-1273 has strong proteolytic, fibrinolytic, and anticoagulant activity in vitro.[3] Considerable purification of this agent, designated as Aspergillin O,[4] has been achieved recently.[5] The present paper illustrates the fibrinolytic and the anticoagulant activity of Aspergillin O in vivo under experimental conditions.

Materials

Three preparations of Aspergillin O of increasing potency were used: an alcohol precipitate,[3] a tannic acid precipitate,* and a more purified acetone precipitate.[5, 6] Since the only variability of effects was related to the difference in concentration of active material in the various preparations, only the results obtained with the more purified acetone material[6] will be described. A pooled preparation was used, with an activity of 800 u. per milligram of lyophilized substance, assayed as described,[4] and dissolved in sterile saline (approximately 1 ml. per milligram of substance). The solution was injected through an unexposed vein of the foreleg at a speed of 3 to 5 ml. per minute. Previous work has shown that Aspergillin O has low toxicity and no antigenicity in dogs.[6]

Methods

These studies were carried out on 70 dogs which were kept under routine laboratory conditions. All procedures were performed under Nembutal or Pentothal sodium anesthesia. The *arterial pressure* was determined during the infusion and for 2 hours thereafter by a direct reading from an Hg manometer connected to a 19 gauge needle lodged in the femoral artery of the animal and kept open by periodic flushing with saline solution. The *temperature* was recorded rectally. *Electrocardiograms* were taken by conventional leads during the infusion.

Thrombi were produced in one of the jugular veins of the animal under study by the intraluminal injection of a 5 per cent sodium morrhuate solution[7] through a small incision of the lower part of the neck. The site of injection was below the point of origin of any large collateral vessel. After a solid clot formed, the incision was closed and the animals were returned to their cages. Twenty-four or 48 hours later, the jugular vein was exposed again 5 to 8 cm. cephalid to the area of the thrombus through a different skin incision. A venogram was then taken with the rapid injection of 4 ml. of 50 per cent Urokon sodium into the vessel. Aspergillin O was administered for a few minutes through a vein of the foreleg. A second venogram was taken 30 minutes after the end of the infusion.

The blood samples were collected in silicone-coated test tubes by means of 17 gauge needles connected to 3 cm. polyethylene tubes before and at various intervals of time after the infusion of Aspergillin O. The following studies were carried out on some or all of the blood samples by the methods referred after each constant: clotting time of recalcified plasma[8]; thromboplastin generation test[9] (in addition to the standard method, a modification was used in which either plasma or serum from the treated animal was matched with serum

*Supplied by the Astra Pharmaceutical Company, Worcester, Mass.

or plasma from an untreated animal. Plasma and serum were diluted 1/25 and 1/10, respectively, with barbital saline buffer. Details of this technique have been given elsewhere[10]; generation of thrombin[11]; activity of antihemophilic globulin[12]; plasma thromboplastic component[13]; and Hageman[14] and Stuart[15] factors. The effect of the addition of $BaSO_4$-absorbed plasma or of native serum from the treated animals on the conversion of purified prothrombin was studied with the method we have described.[1] Essentially, this procedure gives a semiquantitative measurement of the activity of either labile factor (V) or stable factor (VII) and Stuart factor in the conversion of prothrombin to thrombin. The prothrombin activity was studied by the method of Quick[16] on plasma diluted 1/10 with saline solution after addition of an equal volume of 1 per cent solution of bovine fibrinogen in barbital buffer. The fibrinogen level in plasma was studied with a rapid method[17] and with the standard tyrosine method.[16] Both these techniques measure coagulable fibrinogen. Their results were checked against a technique in which fibrinogen was precipitated by 25 per cent saturation with $(NH_4)_2\ SO_4$ and the precipitate reconstituted with saline to the original volume of plasma. After dialysis of the solution against distilled water for 18 hours at 4° C., the nitrogen content was measured by Nessler's method. The comparison of the clotting time of recalcified plasma, rich or poor in platelets, was taken to indicate possible liberation of clotting factors from lysed platelets.[1]

The ability of serum to inhibit the clotting activity of tissue thromboplastin was studied with a standard method.[8] To study the antithrombin activity of serum freshly drawn blood was allowed to clot for one hour at 37° C. The serum was then separated by centrifugation and allowed to stand for 4 hours to obtain complete neutralization of thrombin. Aliquots of 0.1 ml. of serum were mixed with 0.1 ml. of bovine thrombin solution containing 10 National Institutes of Health units per milliliter in 4 test tubes kept in water bath at 37° C. Immediately, or after 10, 30, and 60 minutes of incubation, 0.1 ml. of normal human plasma was added and the clotting time was recorded.

For study of the fibrinolytic mechanism, the blood was collected in a test tube containing 1/10 volume sodium oxalate 0.1 M and the plasma was separated by centrifugation (1,006 RCF for 10 minutes) to make it platelet-poor. The plasma was then tested for fibrinolytic, plasminogen, and antiplasmin activity. The fibrin plate technique of Astrup and Mullertz[18] was used because of our familiarity with this method. Nine milliliters of 0.35 per cent bovine fibrinogen* in barbital buffer at pH 7.4 were transferred to Petri dishes of an average diameter of 10 cm. The solution was then coagulated with the addition of 0.3 ml. of thrombin solution containing 100 National Institutes of Health units per milliliter. The plates were used untreated or after heating at 85° C. for 30 minutes to destroy plasminogen and other components of the fibrinolytic system present in the commercial fibrinogen. To measure the *direct fibrinolytic activity* of plasma the euglobulin fraction was precipitated as described by Kline.[19] The precipitate was dissolved with a volume of barbital buffer equal to that of the plasma originally used. Of the solution, 0.03 ml. was transferred onto heated fibrin plates, which were then incubated at 37° C. for 18 hours. The results are presented as the area of lysis (in mm.^{2}) obtained from the product of measured perpendicular diameters. To test the *plasminogen* (profibrinolysin) *activity*, plasminogen activation was prepared first by mixing equal volumes of streptokinase† (2,100 u. per milliliter) and human‡ euglobulin obtained as above. Five-tenths milliliter of the euglobulin fraction from the treated dog was then incubated one hour at room temperature with 0.1 ml. of human activator, to convert plasminogen to plasmin. Of the mixture, 0.03 ml. was added to a heated fibrin plate and the results calculated as for the fibrinolytic activity. The *antiplasmin activity* was studied by the following method: two-tenths milliliter of plasma was incubated one hour at room temperature with 0.2 ml. of a standard plasmin solution.§ The activity of 0.03 ml. of the mixture on the heated fibrin plate was then tested.

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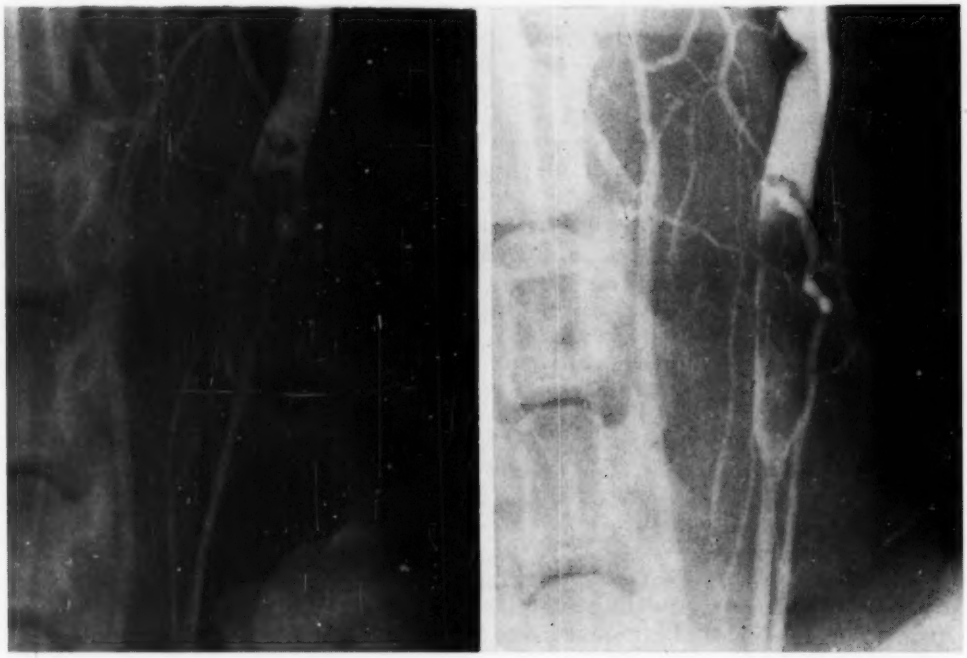
†Varidase, American Cyanamid Co., New York, N. Y.

‡Human plasma was used for the preparation of activator of plasminogen because human plasma, unlike dog and beef plasma, is rich in proactivator.

§Actase, 20 mg. per milliliter.

An alternate method was also used to test the fibrinolytic activity in the circulating blood of the treated animals. Eighteen milliliters of blood was collected in 2 ml. of 0.1 M sodium oxalate by direct venipuncture from one femoral vein exposed in the anesthetized dog. The plasma was separated by centrifugation at 447 RCF for 10 minutes. Aliquots of 0.5 ml. of plasma were pipetted into a series of test tubes and clotted by adding to each tube 20 National Institutes of Health units of bovine thrombin in 0.2 ml. of saline. The clots obtained were allowed to retract at 37° C. for one hour and then washed repeatedly with cold distilled water. Blood was then collected from the same animals at the end of an infusion and 15 minutes and 1, 2, 3, and 4 hours afterward, using 1/10 volume of 0.1 M sodium oxalate as anticoagulant. The plasma was separated by centrifugation. From each sample, 0.5 ml. was diluted with 0.5 ml. barbital buffer. The total mixture was then transferred to a test tube containing a previously prepared homologous plasma clot. A rough estimation of lytic activity was given by the time necessary for complete lysis of the clot during incubation at 37° C. A more quantitative result was obtained when the remaining clot was separated after 3 hours of incubation, washed in distilled water, and tested for fibrin content with the standard tyrosine method.[16]



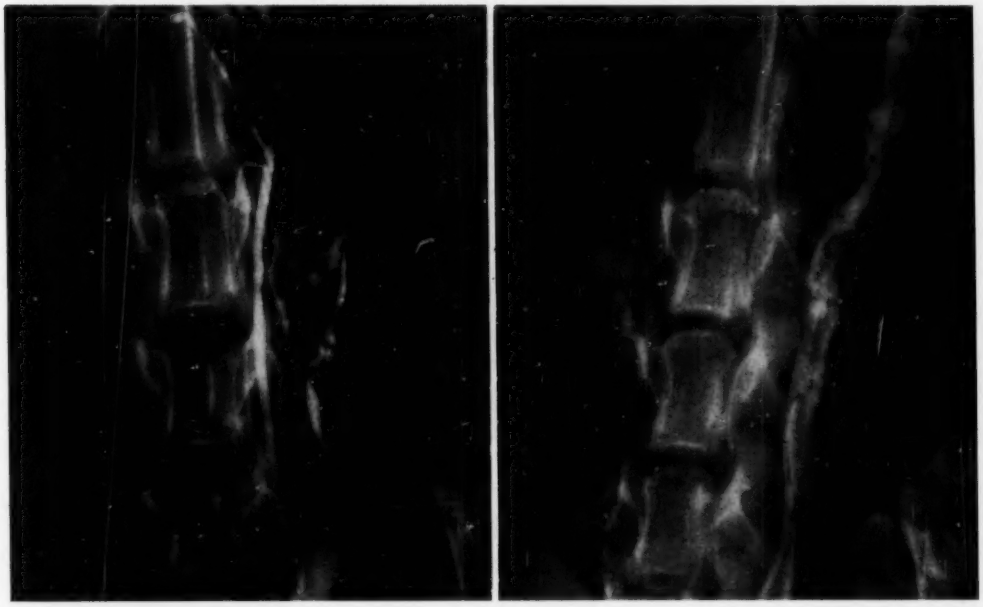
A. *B.*

Fig. 1. Lysis of 24-hour-old sodium morrhuate thrombus by Aspergillin O (8,000 u. per kiloweight). *A*, before infusion. Note the complete obstruction of the main vessel. Blood flow is diverted into a large collateral, just above the point of obstruction. *B*, 30 minutes after infusion. Note recanalization of the main vessel and diminution of the collateral circulation.

Results

The dogs were divided into 3 groups. Detailed studies on the general effects of the infusion of Aspergillin O were conducted in 15 animals. Twenty-five animals were studied for evidence of recanalization of vessels. The effect on

2- and 4-hour-old clots each was studied in 3 animals. The effect on 24-, 48-, and 72-hour-old thrombi was studied in 8, 8, and 3 animals, respectively. The effect of Aspergillin O on various factors and phases of the clotting process was studied in 36 animals.



A. *B.*

Fig. 2. Lysis of 2-day-old sodium morrhuate thrombus by Aspergillin O (8,000 u. per kiloweight). *A*, before infusion. Note the complete obstruction of the main vessel with abundant collateral circulation. *B*, 15 minutes after infusion. Blood now flows at the periphery of the 2-day-old thrombus, outlining its contour. There is re-establishment of the continuity of blood flow in the main vessel.

General effects. No significant changes in rectal temperature, blood pressure, electrocardiographic tracings, R.B.C. and W.B.C. count, blood sedimentation rate, and hematocrit were noted during infusion. Oozing at the site of the surgical incision was noted for one hour after the end of infusion. No bleeding was observed at other sites. Tachycardia occurred if the speed of the infusion exceeded 100 drops per minute.

Evidence of recanalization of thrombosed vessels. Total lysis was obtained when 2- to 4-hour-old clots were challenged with Aspergillin O at doses of 5 mg. per kiloweight or higher. The same dose induced recanalization of veins 24 and 48 hours after establishment of a thrombus in all cases studied (Figs. 1 and 2). The evidence for this conclusion was the re-establishment of the interrupted blood flow, the staining of the surface of the thrombus by the dye, and the diminution of the collateral circulation. Thrombi older than 3 days were unaffected, a finding which is not surprising, since, by such time, organization of the thrombus is well under way.[1]

Table I. Behavior of some clotting factors in 36* animals treated with different doses of Aspergillin O (mean values)

	Dose of Aspergillin O per kiloweight	*Before infusion*	*Time after infusion (minutes)*				
			0	*15*	*60*	*120*	*180*
Clotting time of recalcified plasma (seconds)	2.5 mg.	132.1	97.6	108.2	138.7	142.8	136.6
	5 mg.	127.5	151.7	140.0	132.6	125.2	119.7
	10 mg.	118.3	194.3	166.5	145.9	121.5	128.3
Platelets, 1,000/cm.	2.5 mg.	217.9	184.9	198.8	197.2	200.9	208.1
	5 mg.	194.6	131.3	175.5	198.2	202.7	195.7
	10 mg.	204.2	84.5	146.0	208.0	194.1	201.0
Prothrombin activity (per cent)	2.5 mg.	100.4	98.8	96.6	100.4	100.5	100.0
	5 mg.	100.6	84.2	89.1	97.8	100.7	100.0
	10 mg.	100.2	42.7	33.9	86.5	92.3	98.4
Fibrinogen (per cent)	2.5 mg.	100.0	96.4	100.0	100.5	100.0	100.0
	5 mg.	100.0	72.3	88.1	94.8	99.0	99.8
	10 mg.	100.0	77.3	64.8	57.9	83.0	97.3

*Six animals received 2.5 mg. per kiloweight, 5, 5.0 mg. per kiloweight, and 25, 10.0 mg. per kiloweight.

Table II. The serum antithrombin activity following the infusion of Aspergillin O, with or without the addition of heparin (see text)*

Minutes of incubation of reacting mixture	*Clotting time (seconds)*			
	0	*10*	*30*	*60*
Infusion of Aspergillin O (10 mg. per kiloweight).				
Before infusion	< 3	60	138	180
15 minutes after end of infusion	< 3	22	48	64
2 hours after end of infusion	< 3	40	97	120
3 hours after end of infusion	< 3	57	129	164
Infusion of Aspergillin O (10 mg. per kiloweight) with heparin (0.2 mg. per kiloweight).				
Before infusion	< 3	54	120	174
15 minutes after end of infusion	< 3	62	224	330
2 hours after end of infusion	< 3	57	145	160
3 hours after end of infusion	< 3	52	152	171

*Each figure represents the average of 5 experiments. Note the depression of antithrombin activity after the administration of Aspergillin O, still evident 2 hours later. On the other hand, the addition of small amounts of heparin resulted in considerable increment of antithrombin activity. The increment in antithrombin activity during incubation of the reacting mixture is a constant phenomenon in this test.

Coagulation changes in blood after the administration of Aspergillin O. These were correlated with the dose administered. An average dose of less than 5 mg. per kiloweight accelerated slightly the clotting process in 5 dogs (although still inducing lysis of the clot), as suggested by a moderate shortening of the clotting time and faster generation of thrombin. Results in 36 animals receiving doses of 2.5, 5, and 10 mg. per kiloweight are given in Figs. 3, 4, and 5 and Tables I and II.

RESULTS AT THE END OF THE INFUSION. The platelet count was halved. The clotting time of recalcified plasma was prolonged and the clotting time of platelet-poor plasma after recalcification was significantly longer than that of platelet-rich plasma. The generation of thromboplastin was slowed only moderately. The activity of antihemophilic globulin, plasma thromboplastic component, and Hageman and Stuart factors was not affected appreciably. The plasma prothrombin activity was reduced to approximately one-third. The conversion of purified prothrombin by the accessory factors in the animal plasma and serum was delayed. The generation of thrombin was markedly slowed and incomplete. The fibrinogen level was decreased by an average of 40 per cent.

RESULTS AT 15 MINUTES AFTER THE END OF THE INFUSION. The platelet count was almost back to normal. The clotting time of recalcified plasma was still prolonged in most cases, although occasionally accelerated. The generation of thromboplastin remained moderately slow and the generation of thrombin was still greatly impaired. The fibrinogen and the plasma prothrombin activity

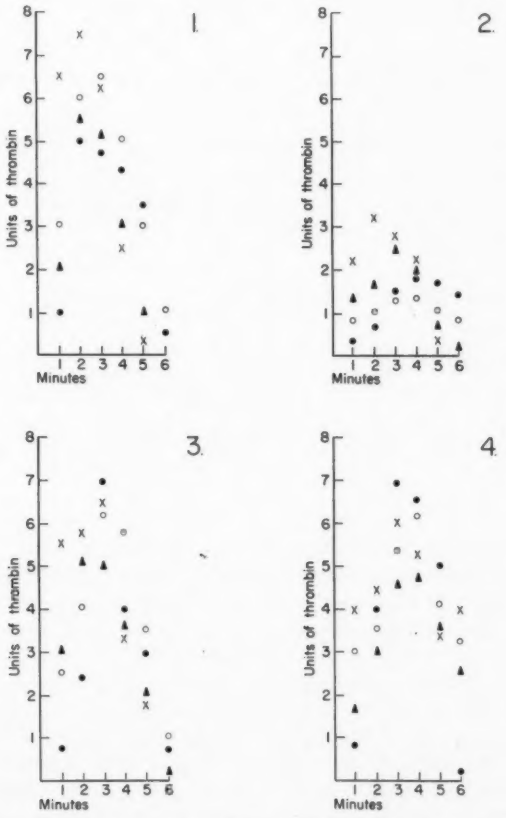


Fig. 3. The generation of thrombin following the infusion of Aspergillin O. *1,* before infusion; *2,* 15 minutes after end of infusion; *3,* 1 hour after end of infusion; *4,* 3 hours afte end of infusion. Points represent individual experiments.

remained at their low level. The conversion of prothrombin stayed delayed when either absorbed plasma or serum from the treated animal was added to purified canine prothrombin. This result indicated that Aspergillin O probably affected the labile (V), stable (VII), and Stuart factors of the conversion of prothrombin.

RESULTS 3 HOURS OR LATER AFTER THE END OF THE INFUSION. Most constants returned to preinfusion values within 3 hours. Often the sample of blood collected one hour after treatment showed faster generation of thromboplastin,

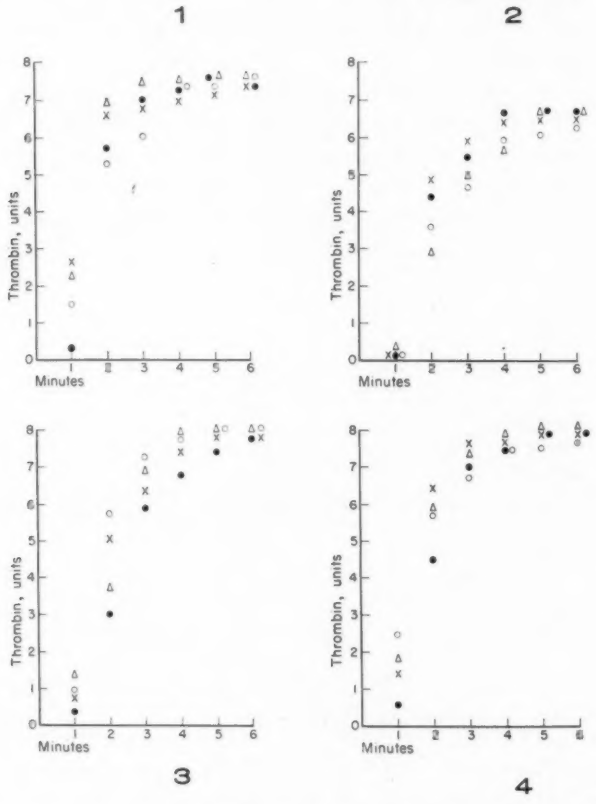


Fig. 4. The effect of adding $BaSO_4$-absorbed plasma from animals receiving 100 mg. of Aspergillin O on the speed of conversion of purified canine prothrombin. (Oxalated plasma from the treated animal was incubated with 100 mg. of $BaSO_4$. After separation it was diluted 1/80 with veronal saline buffer. A mixture was then prepared containing equal volumes of solution of purified canine prothrombin [mg. per milliliter] absorbed plasma and Simplastin and the mixture was incubated at room temperature. After 1, 2, 4, or 6 minutes of incubation, 0.1 ml. of the mixture was transferred to test tubes containing 0.1 ml. of 1 per cent solution of bovine fibrinogen [Armour & Co.] and the clotting time recorded. Values were translated to units of thrombin by means of a standard curve.) *1,* plasma collected before infusion; *2,* plasma collected 15 minutes after end of infusion; *3,* plasma collected 2 hours after end of infusion; *4,* plasma collected 3 hours after end of infusion. Similar results were observed when native serum (diluted 1/80 in saline) was used instead of absorbed plasma. These results would seem to suggest that the delayed formation of thrombin in animals receiving Aspergillin O may be related to depression of the labile and stable factors' activity.

indicating possibly a state of temporary moderate hypercoagulability. The generation of thrombin remained slower than usual. The plasma fibrinogen, although rising, was still slightly subnormal after 6 hours.

The behavior of anticoagulants. The changes in antitissue thromboplastin activity of serum appeared insignificant throughout 3 hours of observation. The serum antithrombin activity showed a sustained decrease after the infusion (Table II). There was, however, an increase in antithrombin activity after incubation of serum and thrombin, possibly partly related to the destruction of fibrinogen and to liberation of by-products with antithrombic activity.[20]

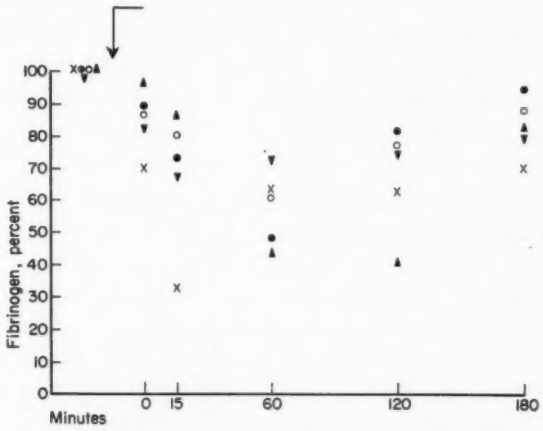


Fig. 5. Effect of the infusion of Aspergillin O on the plasma fibrinogen level (5 representative experiments).

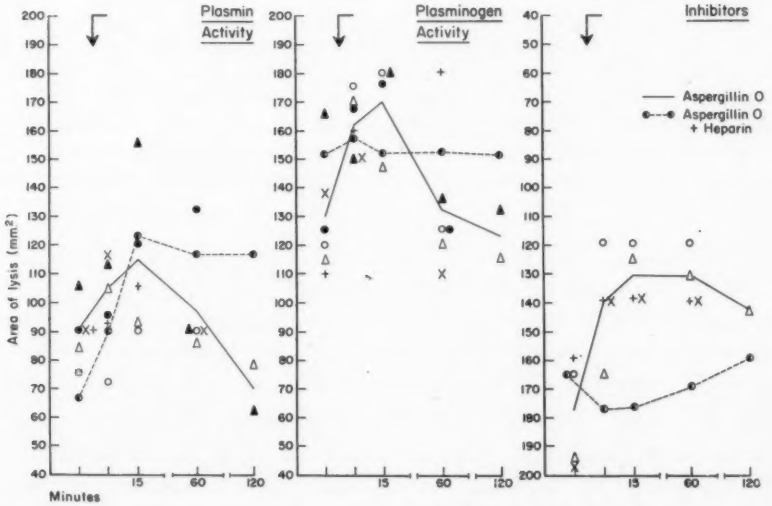


Fig. 6. Plasmin, plasminogen, and antiplasmin activity in the serum of animals receiving Aspergillin O with or without addition of 2 mg. of heparin. (The first point on each group represents the preinfusion values. The lines represent the mean of scattered points and each point indicates a single experiment. The relatively high preinfusion values for plasmin are common with this method.)

The fibrinolytic system during and after infusion of Aspergillin O (Fig. 6). Using the fibrin plate method, the fibrinolytic and plasminogen activities were considerably increased for about one hour. Inhibitors showed higher activity for at least 2 hours. Significant results were obtained when testing the ability of the animal's plasma to lyse preformed fibrin clots. Clots made of homologous plasma were completely dissolved in 8 and 12 hours upon incubation with plasma from dogs which had received 10 and 5 mg. per kiloweight of Aspergillin O, respectively. Maximum lytic activity was present at the end of the infusion and for one and 2 hours thereafter. Lytic activity decreased very rapidly after the second hour, to disappear almost completely after 4 hours. A more accurate quantitative determination of the lytic activity by determination of the residual fibrin in clots after 3 hours of incubation with plasma from experimental dogs showed a similar trend (Fig. 7).

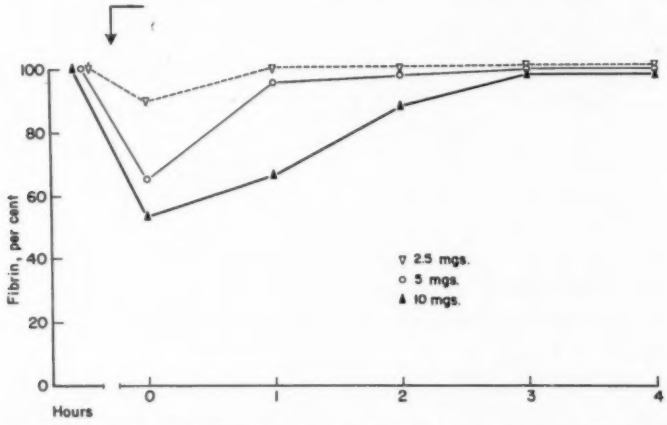


Fig. 7. The semiquantitative estimation of the fibrinolytic activity of plasma of dogs after the infusion of increasing doses of Aspergillin O. Residual fibrin was determined using homologous plasma clots incubated for 3 hours with plasma from the experimental animal. Fibrin levels are expressed in percentages of the initial values. (The greatest activity was present at the end of the infusion and for 2 hours thereafter. It disappeared after 3 hours.) Points represent mean values for 2, 3, and 5 dogs receiving 2.5, 5.0, and 10.0 mg., respectively.

The interaction of heparin and Aspergillin O. Heparin enhances the lytic activity of Aspergillin O on the human fibrin plate and on human plasma clots at a concentration less than 0.2 γ per milliliter, either by decreasing the activity of natural occurring inhibitors of Aspergillin O in plasma and serum or by blocking their activity.[5] It was then felt that the addition of heparin could potentiate the effects of Aspergillin O in vivo. Two milligrams of heparin sodium, together with 100 mg. of Aspergillin O, was infused in 4 dogs. The bleeding at the site of surgical injury was more pronounced and the lytic activity on the thrombi more evident. The drop in platelets was less marked. The changes observed in the clotting mechanism, although similar in type to those seen with Aspergillin O alone, were slightly more pronounced than the latter, with the exception of a sharp

increment in antithrombin activity. Thus it is probable that heparin added its own antithrombin effect to the activity of Aspergillin O. The injection of 2 mg. of heparin in dogs of average size is followed by paradoxical acceleration of the generation of thromboplastin, a finding which may explain the well-known "heparin rebound effect." This did not occur when heparin and Aspergillin O were administered together. There was no increase in plasma antiplasmin activity in animals receiving this combination, and the fibrinolytic activity appeared more sustained (Fig. 6).

Discussion

The evaluation of a potentially thrombolytic drug is obviously a difficult task. Some of the difficulties are inherent to the mechanism of activity of these drugs, at least in our experience.[21] Many available thrombolytic drugs have a biphasic effect. They lyse thrombi at high concentrations, while favoring blood clotting through the accelerated conversion of prothrombin to thrombin at lower concentrations. Thus, most lytic agents could destroy clots first and then hasten formation of new ones by accelerating the formation of thrombin. A second problem is the relatively poor reliability of the methods used in studying the effects of these drugs. Specific examples referring to the material presented in this article refer to the determination of antithrombin and to the study of the fibrinolytic system. The antithrombin test, although often used in coagulation studies, was less than exact, since the plasma used as source of fibrinogen supplies its own antithrombin to the system. As the results are comparative, they were probably significant, and were confirmed on control studies using fibrinogen solution instead of plasma. The meaning of the tests used for the study of the fibrinolytic system in plasma was also questionable. It was not possible to determine whether the euglobulin precipitation technique recovered Aspergillin O as well as plasmin. Thus, direct fibrinolytic and plasminogen activity may have included Aspergillin O in the circulating blood. The antiplasmin activity probably also included the determination of an inhibitor of Aspergillin O. In any event, constant effort was made to keep results on a strictly comparative basis and, for this reason, they were probably valid. Another important obstacle in the evaluation of agents such as Aspergillin O was the determination of its true thrombolytic activity. The destruction of freshly prepared clots was relatively meaningless since fresh clots, not adhering firmly, can be dislodged easily from the vascular wall. Also, retraction, fragmentation, and reduction in size are common in such clots even without drug interference. In our experiments we emphasized the study of 24- or 48-hour-old thrombi since the objections presented for fresh clots possibly did not apply to older thrombi.

It could be concluded, at least in our experimental conditions, that Aspergillin O was a proteolytic and fibrinolytic material* which seemed to possess some advantages over other thrombolytic agents. A state of temporary hypercoagulability was observed with doses too low to induce lysis. At adequate

*It is probably different from the protease isolated by Crewther and Lennox,[22] as described previously.[6]

lytic doses, however, the drug caused a significant and prolonged fall in fibrinogen level, moderate destruction or inhibition of the activity of prothrombin, labile, and stable factors with little effect on the generation of thromboplastin and on the activity of antihemophilic globulin, plasma thromboplastin component, and Hageman and Stuart factors. There was evidence of a considerable delay in the production and of a fall in the yield of thrombin. The blood of animals receiving Aspergillin O, however, also showed a moderate decrease in antithrombin activity, which could be counteracted effectively by the simultaneous administration of heparin. Aspergillin O seemed capable of lysing clots within 48 hours of formation. This conclusion was based on venographic evidence indicating total or partial recanalization of a thrombosed vessel up to 2 days after the induction of experimental venous thrombosis. This type of evidence was made more significant by the observations indicating that increased fibrinolytic activity was detected for at least one hour after administration of Aspergillin O, without decrease in the concentration of plasminogen. Thus, the increased fibrinolysis seemed due, at least in part, to the material injected and not to an activation of the fibrinolytic system. In any case, direct lysis of preformed clots incubated with plasma from the animal receiving Aspergillin O was a clear-cut indication of the ability of the drug to destroy fibrin.

In conclusion, Aspergillin O had a complex action in vivo. It delayed the formation of thrombin, induced hypofibrinogenemia, and, most important, induced lysis of preformed clots. The delay of thrombin formation was limited in time, but might be apparently significant in preventing the formation of new thrombi. The effects of Aspergillin O appeared interesting enough to warrant investigation in the human being.

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Isotopic sodium turnover studies in man: Evidence of minimal sodium (Na^{22}) retention 6 to 11 months after administration

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Na^{22} and a very sensitive whole body counting technique allowed a long term clinical observation in 12 patients who had received small doses of the isotope. Under carefully controlled conditions, the rate of elimination of Na^{22} appeared to be predictably related to NaCl intake. Na^{22} was eliminated in linear fashion until approximately 1 per cent of the original dose remained; thereafter, further elimination of the isotope continued at a decreasing rate. This small and constant final moiety of Na^{22} suggested that the isotope entered the skeletal system, where sodium exchanges slowly.

The use of Na^{22} (physical half-life, 2.6 years) has enabled long-term clinical observations which were not possible with Na^{24} (physical half-life, 15 hours). During the past year Na^{22} has been employed in continuing studies designed to evaluate further the role of sodium in hypertension.[1] This investigation, which employed an extremely sensitive whole body counting technique, allowed an opportunity to make concomitant observations on the retention of Na^{22} in 12 patients who had received small doses of this isotope. It was found that the rate of elimination of Na^{22} was related in a predictable way to NaCl intake. There was remarkably little Na^{22} retention 6 to 11 months after its administration. The small and fairly constant amount of Na^{22} that did remain in the body suggested that the isotope entered a compartment of the body in which sodium exchanges slowly, presumably the skeletal system.

Methods

The adult patients were ambulatory and, though some were hypertensive (see Table I), none had symptoms or signs of cardiac or renal insufficiency. All patients were hospitalized in a metabolic ward and received a constant low sodium diet (0.25 Gm. per day as NaCl) which has been described previously.[2] While in the hospital, the salt intake of the patients ranged from one to 40 Gm. per day, given in the form of enteric, coated tablets. Not all the patients studied received the same salt loads. Repeated urinary analysis of sodium indicated that virtually all of the NaCl in the tablets was absorbed. Only 2 of the patients, R. B. and N. S., were hospitalized continuously, while 9 of the remaining 10,

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after various periods of hospitalization, were discharged on an ad libitum NaCl diet. The exception to this was H. B., who was discharged on a restricted NaCl diet.

Carrier free Na^{22} with a chemical purity of more than 99 per cent was received from Nuclear Science and Engineering Corporation of Pittsburgh, Pennsylvania. The isotope was administered orally in doses of 2.3 to 6.9 μc, with the usual dose ranging between 2 and 4 μc.

A Harshaw NaI 8 inch crystal, connected to three 3 inch photomultiplier tubes, and fed into a Penco 100 channel pulse height analyzer,* was used for counting whole body radioactivity. Na^{22} has an energetic gamma emission with a peak energy of 1.28 mev (channel 60). Because of the excellent counting resolution of the crystal, a window of 1.24 to 1.32 mev. (channels 58 to 62) was used to count body activity. In this range there is a maximum signal to noise ratio. Background was kept to a minimum (30 c.p.m.) by counting patients in a room constructed of 6 inch steel and lined with lead, cadmium, and copper. A fixed contour chair, together with a mechanically positioned crystal, assured a remarkably constant geometry. Repeated countings showed the standard deviation to be less than 1 per cent for standards and patients.

During the first 24 hours, whole body activity varied by as much as 5 per cent without relation to elimination of the isotope. This probably represented internal shifts in the distribution of the isotope as it equilibrated in the body. After 24 hours the whole body activity was reproducible at the 1 per cent level in all patients regardless of salt intake. In estimating whole body activity, the initial counts obtained 24 hours after isotope administration were assigned the value of 100 per cent and subsequent counts were referred to this base.

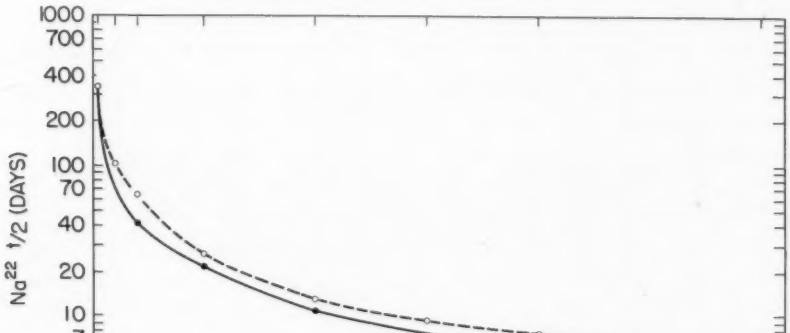

Fig. 1. The effect of different salt intakes on the biologic half-life ($T\frac{1}{2}$) of Na^{22} in patients on a constant diet. The top (dashed) curve refers to patients with essential hypertension. The bottom (solid) curve refers to normotensive subjects. The difference between hypertensive and normotensive subjects on diets containing 2, 5, and 10 Gm. NaCl per day was significant ($p. < .01$). Insufficient observations have been made at other levels to be significant.

Radioactivity in the patients was counted for 5 to 15 minute periods, ordinarily at 1 to 2 day intervals, 6 to 10 times during the course of a given salt load. Counts were corrected for physical decay and plotted, against time, on semi-log paper, as per cent of the original 100 per cent level. The decline in the logarithm of whole body activity with time was linear for a fixed level of NaCl intake, giving a slope which defined the biologic half-lives. When the patients were discharged from the hospital, the calculated amounts of Na^{22} remaining ranged

*Penco, Pacific Electro-Nuclear Corporation, Culver City, Calif.

Table I. Summary of Na^{22} retention and calculated total body irradiation

Patient	*Hospital No.*	*Sex*	*Age (years)*	*Weight (Kg.)*	*Diagnosis*	*Amount of Na^{22} administered (μc)*	*Duration of study (days)*	*Na^{22} retention (at end of study)*		*Calculated total body irradiation* (rads)*
								(%)	*(μc)*	
R. B.	7,159	M	51	83	EHT†	3.6	259	0.21	.008	< 0.2
N. S.	7,741	M	55	63	EHT	3.6	259	0.37	.013	< 0.2
A. K.	6,825	F	55	73	EHT	3.3	322	0.32	.011	< 0.2
B. D.	5,784	F	50	63	N‡	2.3	232	0.27	.006	< 0.2
A. R.	80,019	F	17	58	OB§	6.0	293	0.43	.076	< 0.3
A. E.	80,018	F	51	55	OB	6.9	260	0.14	.010	< 0.3
B. C.	7,094	F	42	51	EHT	2.8	250	0.44	.012	< 0.2
P. R.	5,071	M	58	84	EHT	2.7	198	0.21	.006	< 0.2
M. W.	5,086	F	50	64	N	2.8	173	0.35	.010	< 0.2
R. G.	80,147	F	25	75	OB	2.6	183	0.35	.009	< 0.2
K. W.	80,142	M	63	87	EHT	4.5	203	0.45	.020	< 0.2
H. B.	6,758	M	66	77	EHT	2.6	170	4.51	.117	< 0.3

*Calculated for complete decay of isotope.
†Essential hypertension.
‡Normal.
§Obesity.

from 0.5 to 3.0 μc. At scheduled intervals thereafter they returned for radiation counting until the conclusion of the study.

K^{40} (physical half-life, 1.3 by 10^9 years), which is normally present in the body, was counted before Na^{22} was administered as well as at the end of the study in order that it serve as an internal standard.

Results

Fig. 1 illustrates the biologic half-life ($T\frac{1}{2}$) of Na^{22}, plotted as a function of NaCl intake. From these curves the $T\frac{1}{2}$ for different salt loads can be readily obtained. It can be seen that, on the low sodium diet (0.25 Gm. per day), without NaCl supplements, $T\frac{1}{2}$ was roughly 335 days; as NaCl intake was increased, $T\frac{1}{2}$ decreased, and when intake reached 30 Gm. per day, $T\frac{1}{2}$ was approximately 5 days.

Table I summarizes the data on Na^{22} retention 170 to 322 days after administration. In all patients except H. B., retention of Na^{22} was less than 0.5 per cent of the administered dose. When patient H. B. left the hospital on a restricted NaCl diet it was calculated that 1.6 μc of Na^{22} had not been eliminated. Based on subsequent counting data the estimated average $T\frac{1}{2}$ of Na^{22} outside of the hospital was approximately 33 days. This indicated he had been consuming about 4 Gm. NaCl per day (Fig. 1). This figure was corroborated by analyses of 24 hour urinary sodium samples collected intermittently over a 4 month period.

Table II. Comparison of Na^{22} whole body counts with counts obtained from K^{40}

Patient	*Control count K^{40} (c.p.m.)*	*Initial count of Na^{22}, 24 hours after administration (c.p.m.)*	*Terminal count*	
			Na^{22} (c.p.m.)	*K^{40} (c.p.m.)*
R. B.	43	13,537	29	40
N. S.	32	13,350	50	34
A. K.	24	12,022	38	25
B. D.	33	8,777	24	26
A. R.	22	25,145	108	32
A. E.	27	26,898	39	27
B. C.	20	11,871	53	26
P. R.	39	10,061	21	36
M. W.	24	10,700	37	30
R. G.	27	9,289	33	34
K. W.	42	16,130	73	37
H. B.*	34	9,385	423	

*Patient on restricted NaCl intake (see text). Terminal K^{40} count not possible because of interference from residual Na^{22} energies.

On this intake the Na^{22} retention of Patient H. B. will predictably fall below the 1.0 per cent level in 3 months.* The salt intake of other patients, in whose diet NaCl was not restricted, was estimated from Fig. 1 and corroborated by 24 hour urine sodium excretion to be approximately 10 Gm. per day.

Table II summarizes the control counts for K^{40}, the initial counts for Na^{22}, and the terminal counts for Na^{22} and K^{40}. Although the initial counts were in the range of 3,500 c.p.m. per microcurie of Na^{22} administered, the terminal Na^{22} counts were remarkably low and had the same order of magnitude as K^{40}, which did not vary significantly during the study.

*This prediction has now been verified.

Fig. 2 illustrates the effect of various salt loads on the elimination of Na^{22} in 2 patients studied simultaneously on the same NaCl intakes. The rate of Na^{22} elimination increased directly with NaCl intake. The elimination of Na^{22} was linear for a given NaCl load until the 1 per cent level of retention was approached. Here there was leveling off of the curve for both patients. During this period, supplementary NaCl was discontinued for Patient N. S., and Na^{22} elimination then ceased. In contrast, by continuing Patient R. B.'s NaCl intake at a level of 40 Gm. per day, Na^{22} continued to be eliminated, but at a decreasing rate. This leveling off effect was seen in all of our patients when retention levels approached 1 per cent.

The calculated total body irradition,[3-4] indicated in the last column of Table I, was in each case less than 0.3 rad, and the total body burden at the end of the study was always less than 0.2 μc. This is far below the suggested yearly maximal permissible internal exposure of 5.0 rem[5] and maximum permissible total body burden for Na^{22} of 10 μc recommended by The International Committee on Radiological Protection,[5] and The National Committee on Radiological Protection, respectively.[6] In terms of rads, the residual Na^{22}, considering only its physical half-life of 2.6 years, will be of much less significance than the K^{40} activity, with its physical half-life of 1.3×10^9 years.

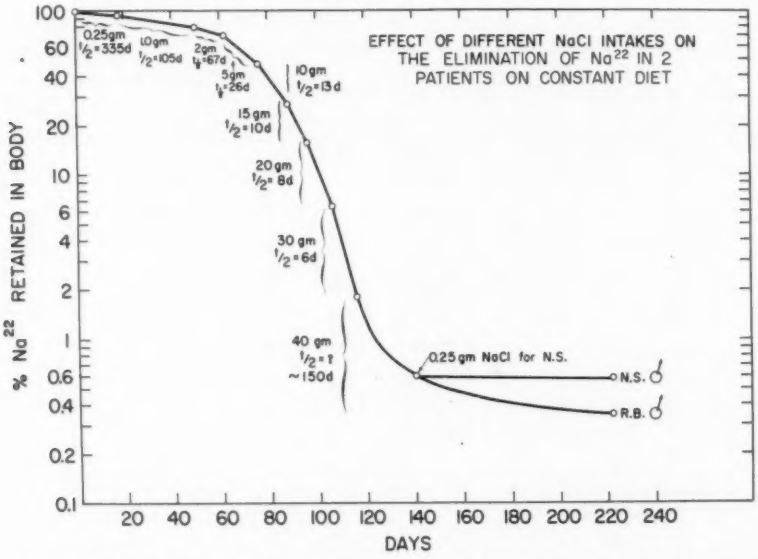

Fig. 2. All bracketed gram values refer to the NaCl intake during the bracketed period. The results were identical for the 2 patients until Patient N. S. was put on a low salt regimen after 140 days (see text).

Discussion

Various reports by such clinical investigators as Miller and associates[7] have indicated Na^{22} retention to be 0.1 per cent after one year. Veall and co-workers[8] reported 1 per cent Na^{22} retention in one human after 75 days. On the basis of our present study, it would seem that differences in retention of Na^{22}, over

a given time, were due primarily to differences in the average daily intake of NaCl. Although a rate difference of Na^{22} elimination between hypertensive and normotensive subjects has been demonstrated,[1] a difference in ultimate retention of the isotope for the two groups was not discerned. The rate of Na^{22} elimination for different salt intakes was predictable and the extremes were quite great. For example, on the low sodium intake used here, the $T\frac{1}{2}$ was found to approximate 1 year, thus it would require roughly 7 years to reach the 1 per cent level of retention. On the other hand, if a patient were given 30 Gm. of NaCl per day, the $T\frac{1}{2}$ of Na^{22} would be about 5 days and 99 per cent of a tracer dose of Na^{22} would be eliminated in approximately 35 days (Fig. 1). Normally, an individual on an average intake of NaCl (10 Gm. per day) will eliminate 99 per cent of a given dose of Na^{22} within 4 months. At approximately the 1 per cent level of retention, linear elimination of Na^{22} was found to level off asymptotically even with a high salt load and, thereafter, elimination of Na^{22} was estimated to have a $T\frac{1}{2}$ averaging 150 days over the next several months (Fig. 2). Beyond one year, further elimination of Na^{22} can be estimated conservatively in terms of its physical half-life.

The leveling off of the retention curve could be explained if the remaining Na^{22} was concentrated in a compartment such as the skeletal system, where sodium exchanges slowly. Approximately one third of total body sodium is in the skeleton,[9-11] and there is increasing evidence that at least some of this sodium participates actively in metabolic processes.[12-14] However, it is not clear whether all of the sodium is easily exchangeable with Na^{22}. Green and associates[15] found that after 14 weeks there was almost complete equilibrium of Na^{22} in rats, whereas Edelman and co-workers[16] found that after 4 hours about 45 per cent of bone sodium in dogs exchanged with Na^{22} and that there was no increased exchange during the next 4 weeks. Na^{22} exchange in bone is extremely complex, and subject to considerable variation.[17-22] In any event, it appears that some of the bone sodium, presumably that contained in the crystal lattice,[23, 24] exchanges slowly. This is the most likely repository of the final 1 per cent of Na^{22} observed in the present studies.

In patients on average salt intake diets who had received less than 5 μc of Na^{22} it was calculated that total body irradiation, allowing for infinite time, would be less than 0.3 rad. The Na^{22} remaining in the body 6 to 11 months after administration of the isotope approximated .01 to .02 μc. This is a very slight amount of radioactivity, but present evaluation of the ultimate effects of small point radiation sources is incomplete. We consider the danger to be insignificant. However, since there is a potential hazard, we would recommend doses of less than 5 μc of Na^{22} for clinical studies.

We wish to thank Dr. Stanton Cohn and Mr. Michael Stravino for their assistance in the whole body counting procedure.

Addendum. Subsequent observations on 5 patients (R. B., N. S., A. K., B. C., and P. R.) 15 to 21 months after isotope administration indicate that the small amount of Na^{22} remaining in the body continues to exchange at an increasingly slower rate. Therefore, it appears that if sufficient time is allowed for exchange, there will be essentially no permanently bound Na^{22} in the adult.

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Reproducibility and reliability of the Schilling test

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The reproducibility and reliability of the Schilling test in the same individual have been assessed by a study involving repeated Schilling tests in 7 patients with pernicious anemia, 2 patients who had undergone total gastrectomy, and one patient with adult celiac disease. The test was found to be reliable and reproducible in demonstrating malabsorption of Co^{58}-labeled vitamin B_{12} in patients with pernicious anemia and in those who had undergone a total gastrectomy; a wide range of results was obtained in the patient with celiac disease.

When Schilling tests were performed with intrinsic factor in the patients with pernicious anemia and in those who had undergone total gastrectomy there was evidence that the effect differed not only from patient to patient but also in the same patient at different times. The test with intrinsic factor was not reliable unless the dose of the factor was adequate. Difficulties of determining an adequate dose of intrinsic factor are outlined and illustrated by the results. There appeared to be an absorption defect in patients who had undergone total gastrectomy which differs from that found in patients with pernicious anemia. Oral calcium did not increase the absorption of vitamin B_{12} in the patient with celiac disease.

Deficiency of vitamin B_{12} in man can occur from a variety of causes, the most common in temperate climates being diminished absorption from the alimentary tract. The capacity to absorb vitamin B_{12} can be estimated with any degree of accuracy only by use of the radioactive vitamin in absorption studies. These tests are of considerable assistance in defining the nature of the defect and are often the only means of establishment of diagnosis in patients who have received treatment.

Of the methods used to investigate the absorption of radioactive vitamin B_{12}, the urinary excretion test, introduced by Schilling,[5] is the most convenient and thus has found wide acceptance. Briefly, this test entails administration

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of an oral dose of radioactive vitamin B_{12} followed by an intramuscular injection of the nonradioactive vitamin; the greater part of the intramuscular dose is excreted in the urine and "carries out" some of the radioactive vitamin B_{12} which has been absorbed from the intestine. The amount of radioactivity in the urine over the next 24 hours thus gives an indication of the amount of the radioactive vitamin B_{12} absorbed from the intestine.

We have used this test on over a thousand occasions, and, in general, our experience has been in keeping with published results. On a few occasions, however, anomalous results were obtained which could not be ascribed to technical errors; this applied particularly to tests used in a semiquantitative manner. Reference to the literature showed that little attention had been paid to the reproducibility of the test. For this reason a study designed to yield information about the reproducibility and reliability of the test was carried out, the results of which are reported in this paper.

Materials and methods

Ten patients were studied. The diagnosis of Addisonian pernicious anemia had been established in 7 patients (Cases 1 through 7) on the basis of macrocytic anemia, megaloblastic erythropoiesis seen on marrow biopsy, a histamine-fast achlorhydria test, low serum vitamin B_{12} level, and a response to parenteral vitamin B_{12}. Two patients (Cases 8 and 9) had undergone total gastrectomy and presented with a macrocytic anemia, megaloblastic erythropoiesis seen on marrow biopsy, and a low serum vitamin B_{12} level; they also responded to parenteral vitamin B_{12}. One patient (Case 10) with a typical case of adult celiac disease came under observation with a macrocytic anemia and megaloblastic erythropoiesis seen on marrow biopsy which failed to respond to vitamin B_{12} but responded to oral folic acid; fat balance showed gross steatorrhea. All the patients had been treated with parenteral vitamin B_{12} prior to this investigation and, in addition, Case 10 continued to take oral folic acid and a gluten-free diet during the tests. All were aware of the nature and objects of the investigation and were willing participants.

*Radioactive Co^{58} vitamin B_{12}.** This was diluted to a working concentration of 0.5 μg, specific activity 0.5 μc, in 5 ml. saline. The solution was dispensed in acid-washed dark glass bottles, autoclaved, and stored at 4° C. when not in use. Several batches were used in the course of the trial. The vitamin B_{12} content of each batch was checked by microbiologic assay at weekly intervals.

Schilling tests. The tests usually were carried out at intervals of one week or longer. They were performed with and without intrinsic factor and, as far as possible, these procedures were used alternately. Some patients fasted overnight and some had a tea and toast breakfast 2 to 3 hours before the test; whichever regime the patients preferred to adopt was followed for every test. The oral dose of Co^{58}-labeled vitamin B_{12} was given in 200 ml. water at 9:30 A.M. and 1,000 μg vitamin B_{12} was injected intramuscularly 2 hours later. The intrinsic factor was dispensed in lots of 15 mg. and was suspended in the Co^{58}-labeled vitamin B_{12} solution a few minutes before ingestion. The same batch of intrinsic factor was used throughout the investigation. All urine passed in the 24 hours after the injection was collected and there was every reason to believe that the collections were complete.

The total urine volume was measured, shaken vigorously, and taken into plastic bottles in 450 ml. aliquots. The radioactivity in the aliquots was measured by the end-on method in a suitably modified Ekco scintillation counter N 550 with a thallium-activated sodium iodide crystal 3.81 cm. in diameter and 2.4 cm. deep, shielded by 3 inches of lead, and an Ekco automatic scaler, type N 530 A. A standard was used containing 0.2 μc Co^{58}-labeled vitamin B_{12}, with 2,000 μg vitamin B_{12} as a carrier in 450 ml. water. All samples were counted for 300

*The Radiochemical Centre, Amersham, England.

seconds. The background count ranged from 1.49 to 4.95 counts per second, and the net standard counts from 124 to 194 counts per second. When the gross counts per sample were less than twice the background count the radioactivity present was regarded as insignificant, and a value of 0 per cent of the dose excreted was recorded. It will be seen that a coincidence of a high urine volume, a low urine radioactivity, a high background count rate, and a low standard count rate might result in a recorded figure of 0 per cent of dose excreted when in fact the actual figure might have been 5 per cent. In fact, this combination of circumstances did not arise in practice, and all values recorded as 0 per cent of dose excreted represent true figures of less than 2 per cent of dose excreted in all cases and less than 1 per cent of the dose excreted in the majority of results. As the object of the investigation was to assess the test in routine clinical practice, the dose of radioactivity was not increased to ensure a degree of accuracy in results which we felt to be unrealistic.

Six Schilling tests were carried out in all patients and the same number of tests with a single dose (15 mg.) of intrinsic factor were carried out in the patients with pernicious anemia and postgastrectomy megaloblastic anemia. Two of the patients with pernicious anemia (Cases 6 and 7) were further studied for observation of the effect of double (30 mg.) and quadruple (60 mg.) doses of intrinsic factor. The effect of adding 70 ml. of 10 per cent calcium gluconate to the radioactive vitamin B_{12} was studied on 6 occasions in Case 10.

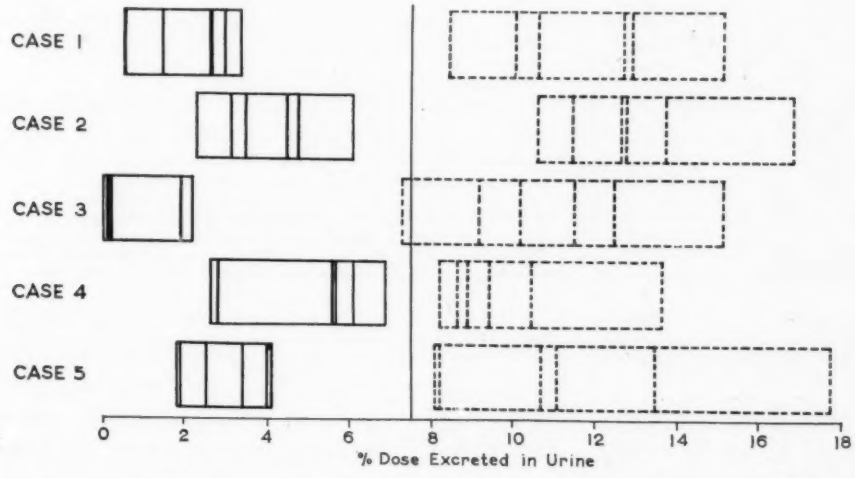

Fig. 1. Results of 6 Schilling tests alone (|) and 6 with a single dose of intrinsic factor (¦) in each of 5 patients with pernicious anemia (Cases 1 through 5). The vertical line at 7.5 per cent dose excreted in the urine delineates the abnormal from the normal results.

Results and analysis

The results are recorded in diagrammatic form in Figs. 1 through 3 and the details are given in Tables I through IV.

Patients with pernicious anemia. In these patients (Cases 1 through 7) the results of the Schilling tests range from 0 to 6.9 per cent, agreeing with the accepted range of 0 to 7.5 per cent.[4] Because of the small number of patients in this group it was considered inadvisable to carry out further comparisons with other published results.

When the tests were performed with a single dose of intrinsic factor in Cases 1 through 7 the mean values of the 6 tests in each patient were all greater than 7.5 per cent and significantly different ($P < 0.01$) from the mean values of

Table I. Mean values of Schilling tests with and without single dose of intrinsic factor in 7 patients with pernicious anemia (Cases 1 through 7) and 2 patients after they had undergone total gastrectomy (Cases 8 and 9)

Case No.	Schilling tests (% dose excreted)			
	Without intrinsic factor		With intrinsic factor	
	Mean	Standard deviation	Mean	Standard deviation
1	2.3	1.1	11.7	2.4
2	4.1	1.4	13.1	2.2
3	0.7	1.1	11.0	2.8
4	4.9	1.8	9.9	2.0
5	2.9	1.2	11.6	3.7
6	1.7	1.6	8.0	3.0
7	1.4	1.2	9.7	3.3
8	1.5	1.6	7.5	3.4
9	2.4	1.2	6.5	3.4

Each value is derived from 6 observations without intrinsic factor and 6 observations with a single dose of intrinsic factor on each patient.

Table II. Mean values of Schilling tests with double and quadruple doses of intrinsic factor in 2 patients with pernicious anemia (Cases 6 and 7)

Case No.	Schilling tests (% dose excreted)			
	With double dose intrinsic factor		With quadruple dose intrinsic factor	
	Mean	Standard deviation	Mean	Standard deviation
6	9.4	1.0	20.0	2.8
7	11.1	4.1	14.1	4.2

Each value is derived from the results of 6 observations with each dose of intrinsic factor on each patient.

the tests without intrinsic factor in the same patients. Nevertheless, out of the 42 tests in which a single dose of intrinsic factor was given, 4 subjects had values of less than 7.5 per cent—Case 3, 7.3 per cent; Case 6, 2.8 and 6.2 per cent; Case 7, 4.3 per cent. When Cases 6 and 7 were tested with a double dose of intrinsic factor the mean values of the 6 tests in each patient were slightly, but not significantly, greater than the mean values of the tests with a single dose of intrinsic factor, and one of the 12 values was less than 7.5 per cent (Case 6, 4.2 per cent). When a quadruple dose of intrinsic factor was used the mean values of the 6 tests in Case 6 were significantly greater ($P < 0.01$) than the mean values of the tests with a single or double dose of intrinsic factor; Case 7 also showed an increase in the mean values of the 6 tests, but this was not significantly different from the mean values of the tests with a single or double dose of intrinsic factor. No individual result below 7.5 per cent was found when a quadruple dose of intrinsic factor was used. An analysis of variance shows that Cases 6 and 7 reacted in significantly different ways to the increasing doses of intrinsic factor—Case 6 had a smaller mean value for the tests with a single and double dose of intrinsic factor than did Case 7, but had a higher mean value in the tests with a quadruple dose of intrinsic factor.

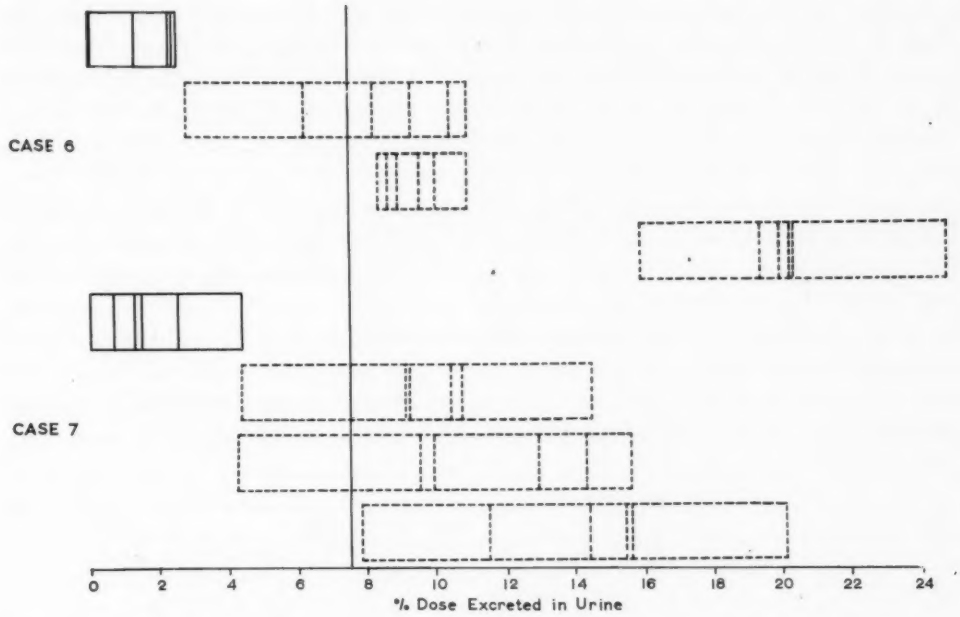

Fig. 2. The results of 6 Schilling tests alone (|) and 6 each with a single, double, and quadruple dose of intrinsic factor (|) in 2 patients with pernicious anemia (Cases 6 and 7). The uppermost results represent the single dose of intrinsic factor, the lowermost, the quadruple dose of intrinsic factor in each case. The vertical line at 7.5 per cent dose excreted in the urine delineates the abnormal from the normal results.

Table III. Mean values of Schilling tests with and without intrinsic factor in 7 patients with pernicious anemia (Cases 1 through 7) and in 2 patients after they had undergone total gastrectomy (Cases 8 and 9)

	Schilling tests (% dose excreted)	
	Without intrinsic factor	*With single dose of intrinsic factor*
Cases	*Mean*	*Mean*
1-7	2.57	10.70
8 and 9	1.92	6.98

Each value is derived from 6 observations without intrinsic factor and 6 observations with a single dose of intrinsic factor on each patient.

Table IV. Mean values of Schilling tests with and without oral calcium in a patient with celiac disease

	Schilling tests (% dose excreted)			
	Without calcium		*With calcium*	
Case	*Mean*	*Standard deviation*	*Mean*	*Standard deviation*
10	5.2	3.8	4.9	3.2

Each value is derived from 6 observations without calcium and 6 observations with calcium.

Patients after performance of total gastrectomy. The results of the Schilling tests in the 2 patients who had undergone total gastrectomy (Cases 8 and 9) are in keeping with the values obtained in Cases 1 through 7, and the mean values of the 2 groups are not significantly different. With a single dose of intrinsic factor Cases 8 and 9 each had 3 results greater and 3 results less than 7.5 per cent. The mean values were 7.5 per cent in Case 8 and 6.5 per cent in Case 9: there is a highly significant difference ($P < 0.01$) between the mean values of the tests with a single dose of intrinsic factor in the patients with pernicious anemia (Cases 1 through 7) and the patients who had undergone total gastrectomy (Cases 8 and 9).

The patient with celiac disease. The 6 Schilling tests in Case 10 gave a wide range of results. Three were greater and 3 less than 7.5 per cent, the mean value being subnormal at 5.2 per cent. When the tests were performed with calcium gluconate the mean value was 4.9 per cent and only one result was greater than 7.5 per cent.

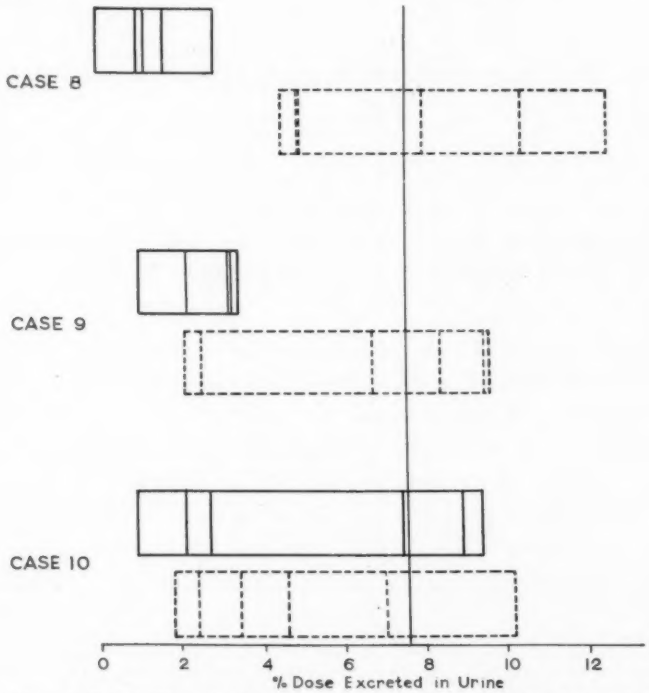

Fig. 3. The results of 6 Schilling tests alone (|), 6 with a single dose of intrinsic factor (¦) in 2 patients after total gastrectomy (Cases 8 and 9), and with oral calcium (¦) in a patient with celiac disease (Case 10). The vertical line at 7.5 per cent dose excreted in the urine delineates the abnormal from the normal results.

Particular attention was paid to the possibility of a progressive increase or decrease in the excretion of urine radioactivity in individual patients during the investigation for two reasons. First, because it was possible that the patients had become refractory to the intrinsic factor used, and second, because the repeated injections of vitamin B_{12} might have modified the excretion of

radioactivity over the period of the investigation. This did not occur, and there was no evidence of a significant rise or fall, over the period of investigation, in the urine radioactivity whether the tests were performed alone, with intrinsic factor, or with calcium gluconate.

Discussion

The conduct of the test is essentially that described by Schilling.[5] The term "Schilling test" is used loosely, as modifications have been introduced by several writers, notably in respect of the mass of radioactive vitamin B_{12} given orally and the mass and timing of the intramuscular vitamin B_{12}. The results probably depend on these factors[4] and it must be stressed that our results and interpretations can only be compared with results from tests conducted on similar lines.

Our results show that the test is reproducible and confirm the view that the Schilling test is a reliable method of detecting malabsorption of vitamin B_{12} in patients with Addisonian pernicious anemia and in those who have undergone total gastrectomy. The results in the patient with celiac disease suggest that a single Schilling test may not be a reliable or reproducible measure of the patient's ability to absorb vitamin B_{12} in this disease and that repeated tests may be necessary to assess this function.

The results of the tests with intrinsic factor in the patients with pernicious anemia are interesting. The single dose of intrinsic factor was adequate in that it produced a normal mean value in all patients, but there were 4 misleading or "false negative" individual results out of the 42 tests. Three of these "false negative" results were obtained in 2 patients who were then further investigated with a double and quadruple dose. When the double dose was used the mean value was again normal, but one of the 12 individual results was misleading. When a quadruple dose was used there were no misleading individual values in 12 tests. These findings raise the possibility that the effect of intrinsic factor differs not only from patient to patient but also in the same patient at different times. The results also suggest that the test would be entirely reliable provided that the dose of intrinsic factor is adequate. The difficulty of assessing the adequacy of a dose of intrinsic factor is clearly demonstrated by the results and it follows that repeated testing with intrinsic factor may be necessary to establish a diagnosis of intrinsic factor deficiency. We accepted values of less than 7.5 per cent dose excreted when intrinsic factor was given as misleading or false negative results because we believe that, in clinical practice, it is advisable to have a definite, if rather arbitrary, value to delineate the normal from the subnormal result. It could be argued that such an attitude is unnecessarily rigid and that all that is required to demonstrate the effect of intrinsic factor is a significant rise in the excretion of urinary radioactivity when intrinsic factor is added, in which case some of these results might not be classified as misleading. However, the problem of deciding whether or not an increase is significant is obviously extremely difficult in any one case, and we prefer to adopt the former course, believing it to be less liable to

misinterpretation and more satisfactory in clinical practice. In making this decision we are much influenced by the range of values obtained in Cases 1 through 7, when the tests were done without intrinsic factor.

The Schilling test is often used in a quantitative or semiquantitative manner in assay of intrinsic factor preparations. This has its basis in the observation[2] that there is a correlation between the amount of radioactivity excreted in the urine and the amount actually absorbed, as deduced from fecal collection of the unabsorbed radioactive vitamin B_{12}. Our results suggest that the test may be of doubtful value when used in this manner. The wide range of values obtained in the tests with a single dose of intrinsic factor in each individual shows how misleading the results of a single test can be if interpreted quantitatively, and the finding that there is no significant difference between the mean values of the tests with a single dose of intrinsic factor and those with a double dose, as in Cases 6 and 7, is good evidence of this. Additional evidence is the nature of the response in Cases 6 and 7 to increased doses of intrinsic factor.

In presenting these facts it is not necessarily implied that they are to be taken as evidence against there being a stoichiometric relationship between the amount of B_{12} absorbed and the amount of intrinsic factor given.[1] The evidence for this relationship was drawn from the results of fecal excretion studies. It would be unwise to compare the results obtained by very different methods, particularly when one is an indirect method of assessing absorption. It is suggested, however, that further study of this point is desirable.

The results obtained with the patients who had undergone total gastrectomy are particularly interesting in relationship to those found with the patients with pernicious anemia. Theoretically, these patients have an absorption defect comparable to that seen in pernicious anemia, and the defect should be rectified by intrinsic factor. In 5 out of 12 tests with a single dose of intrinsic factor, however, the absorption defect was not corrected, and the mean values for these patients were significantly less than those for the patients with pernicious anemia. This cannot be regarded as a serious diagnostic problem, but it is evidence that the absorption defect in such patients is not entirely comparable to that seen in pernicious anemia. It is possible that these patients have a greater need for intrinsic factor than patients with pernicious anemia, but preliminary investigations do not suggest this view. Further studies on this subject are in progress.

Comment has already been made on the wide scattering of results in Case 10. In our experience this case is not exceptional, and an important diagnostic error may arise as a result of this phenomenon unless this is borne in mind. The error arises as follows: a patient is tested and a value of less than 7.5 per cent is obtained, a second test is done with intrinsic factor and a value of more than 7.5 per cent is obtained, and the diagnosis of pernicious anemia is made. Such results could of course occur in patients with celiac disease, whether intrinsic factor were added or not, and the error will not be discovered unless repeated tests are performed. Experience has shown us that this error does occur in practice. The lack of response when the tests are repeated with oral

calcium is disappointing in view of the report suggesting that calcium augments the absorption of B_{12} in celiac patients.[3] The wide range of values obtained on this patient might be explained on the basis that the patient's ability to absorb vitamin B_{12} was changing from test to test. Whether this is the explanation for the results or whether the results simply show that the test is not reproducible, it is obvious that a single test is unlikely to be a valid measure of such a patient's ability to absorb vitamin B_{12}. Repeated tests are probably necessary to assess this function.

When the patients with pernicious anemia were tested with intrinsic factor there was evidence that the effect differed not only from patient to patient but also in the same patient at different times. The test with intrinsic factor was reliable only if the dose was adequate, and there is some difficulty in assessment of this. The test was of little value when used in a quantitative manner.

The absorption defect seen in the 2 patients who had undergone total gastrectomy differed from that found in the patients with pernicious anemia.

A wide range of results was obtained in the patient with adult celiac disease and oral calcium did not increase the absorption of vitamin B_{12}. The implication of this finding has been described.

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The in vitro incorporation of nucleic acid precursors into leukemic human leukocytes. I. Methodology and effects of therapy on incorporation in vitro

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Methods are described for an assay system utilizing the in vitro incorporation of several nucleic acid precursors into nucleic acid bases of leukemic human leukocytes. Values for the incorporation of adenine-8-C^{14} into 2 nonleukemic patients, 3 with chronic lymphatic leukemia, and 7 with chronic granulocytic leukemia are given and compared with their differentials. The variation in incorporation of 3 precursors by leukocytes drawn from 2 leukemic patients before and after x-radiation was determined. Specific activities did not change markedly until there was a definite drop in total white count and significant changes in the differential to more mature forms. There were then small decreases in specific activities of ribonucleic acid bases and large decreases in those of deoxyribonucleic acid bases.

We have investigated the incorporation of various nucleic acid precursors into human leukemic white blood cells in vitro for two purposes: first, to attempt to develop an assay system which could be used to test the action of therapeutic agents in vitro, and to test the effects of in vivo therapy on peripheral white blood cells (if these effects were manifested in vitro); second, to investigate some aspects of nucleic acid metabolism in these cells, with the idea of using this information in studies of resistance to certain agents used in treatment of leukemia. The more detailed studies of nucleic acid metabolism will be published elsewhere.[1] In the present manuscript, we wish to describe an assay system and its use in measuring the variation in incorporation by white blood cells drawn before and after roentgen treatment of leukemic patients. In a subsequent manuscript, we shall tabulate the effects of various chemotherapeutic agents in vitro.

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Methods

Preparation and incubation of leukocyte suspension. Heparinized blood was removed by venipuncture from leukemic patients having white blood counts of 75,000 to 400,000 cells per cubic millimeter. The blood was allowed to settle at 3° C. for 1 hour, after which the leukocyte-plasma layer was removed by aspiration and centrifuged for 5 minutes in a clinical centrifuge at 1,470 g. The cells were washed in saline, thrice their volume, centrifuged, and suspended in Robinson's salts mixture[2] supplemented with 100 mg. per cent glucose and 209 mg. per cent sodium bicarbonate. Duplicate 60 ml. Warburg vessels, each containing 4 to 6 by 10^8 cells in 12 ml. medium plus radioactive precursor, were incubated at 38° C. in a 95 per cent air: 5 per cent carbon dioxide atmosphere. The incubation was stopped at 30 to 240 minutes by removal of the flasks to an ice bath and the addition of 1 ml. of 2.6 M perchloric acid (PCA).

Separation of acid-soluble and nucleic acid fractions. Operations up to the extraction of nucleates in hot NaCl were performed in the cold. Extractions were carried out in 15 ml. centrifuge tubes with centrifugation in a clinical centrifuge at 1,470 g. The contents of the flasks were centrifuged for 5 minutes and the acid-insoluble residues were washed 2 times with 2 ml. portions of cold 0.2 M PCA. In some studies the acid-soluble supernates from the first extraction and wash were pooled for analysis of acid-soluble compounds.

The extraction of sodium nucleates from the acid-insoluble residue and the separation of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were essentially as described by Hurlbert and Potter.[3] The acid-insoluble centrifugate was suspended in 1.0 ml. 10 per cent NaCl plus 1 drop 0.4 per cent phenol red, and adjusted to the color of Merthiolate with NaOH made up in 10 per cent NaCl. Adjustments of pH were made in an ice bath and back-titrated with HCl made up in 10 per cent NaCl, if necessary. After centrifugation for 5 minutes, the centrifugate was extracted with 2.0 ml. 10 per cent NaCl for 30 minutes at 100° C. and centrifuged for 10 minutes. The centrifugate was re-extracted for 30 minutes at 100° C. The supernates from the 3 extractions were pooled in a centrifuge tube and chilled. Ninety-five per cent ethyl alcohol, thrice the volume of the supernates, was added, and the mixture was allowed to stand 4 hours or longer in the cold.

The precipitated sodium nucleates were centrifuged, suspended in 0.5 ml. of 0.1 N NaOH, and incubated 16 hours at 38° C. The tubes were then chilled, and 0.35 ml. H_2O plus 0.15 ml. of 1.0 N HCl was added. The DNA precipitate was centrifuged in the cold and washed once with 0.5 ml. 0.1 N HCl. This wash was added to the RNA supernate. The DNA precipitate was redissolved in 0.5 ml. of 0.1 N NaOH and heated at 80° C. for 20 minutes. The precipitation of DNA was carried out as before, but this time the 0.1 N HCl was discarded.

Isolation of purine bases of RNA and DNA. The DNA precipitate was suspended in 1.0 ml. of 1.0 N HCl and the RNA supernate was made 1.0 N, with respect to HCl, by adding 0.15 ml. 10 N HCl and making the volume 1.5 ml. with H_2O. The tubes were then heated for 1 hour at 100° C., cooled, diluted to 4.0 ml. with H_2O, and poured on 0.8 by 1.5 cm. Dowex-50 columns. To remove pyrimidine nucleotides, the following fractions were allowed to drip through and were discarded: sample, 3 ml. H_2O rinse of tube, 2 ml. 1.0 N HCl. The purine bases were then eluted with 4 ml. 3.0 N HCl (guanine) and 6 ml. 6.0 N HCl (adenine), collected in 10 ml. beakers, and taken to dryness in a vacuum desiccator at room temperature. The contents of the beakers were taken up in 0.1 ml. 0.1 N HCl, spotted on Whatman No. 1 paper, and chromatographed by descending chromatography on the isoamyl-disodium phosphate system of Carter.[4] The purines were detected under ultraviolet light and the spots were cut out and eluted in HCl at 37° C. as follows: adenine in two 2 ml. portions of .01 N HCl for 1 hour and for 30 minutes; guanine in 1 ml. 0.1 N HCl for 2 hours, and then in 2.5 ml. .01 N HCl for 1 hour. The quantity of base present was determined by measurement of ultraviolet absorption in the Beckman spectrophotometer, and suitable aliquots (usually 0.5 ml.) were plated on aluminum planchets for measurement of radioactivity in a flow counter.

Isolation of pyrimidine nucleotides of RNA and pyrimidine bases of DNA. The procedure was the same as for the isolation of purines, through the method of separation of RNA and DNA. Methods for RNA were essentially as described by Edmonds and LePage.[5] The RNA supernatant was hydrolyzed and poured on Dowex-50 columns as described for purines. The sample effluent, a 2 ml. H_2O fraction, and a 4 ml. 2 N HCl fraction were collected for each sample in a 20 ml. beaker. This treatment removed pyrimidine nucleotides but left adenine and most of the guanine on the column. The subsequent procedure was the same as for purines, except that the isopropyl-HCl system of Wyatt[6] was used for paper chromatography.

Seventy per cent perchloric acid, 0.2 ml., was added to each tube of DNA precipitate.[6] The tubes were heated at 100° C. for 60 minutes, cooled, and diluted to 0.5 ml. with water. They were placed in an ice bath, and 10 N KOH was added in drops until no more precipitate formed. The samples were then centrifuged and the supernatants were removed by aspiration, made up to 4 ml. with water, and poured on 0.8 by 2.0 cm. Dowex-50 columns. The subsequent procedure was the same as for RNA pyrimidines.

Isolation of DNA from thymidine-H^3 incorporations. The precipitated sodium nucleates (see above) were dissolved in 0.5 ml. 0.1 N NaOH and heated 20 minutes at 80° C. The tubes were chilled, and 0.35 ml. H_2O plus 0.15 ml. 1.0 N HCl was added. The DNA precipitate was centrifuged 10 minutes in the cold and washed once with 0.5 ml. 0.1 N HCl. It was then dissolved in 0.5 ml. concentrated formic acid; 0.2 ml. was pipetted into a tube for DNA determinations by the Dische diphenylamine method,[7] and 0.2 ml. was plated on an aluminum planchet for measurement of radioactivity in a flow counter.

Isolation of protein from glycine-2-C^{14} incorporations. After the initial extraction and 2 washes with 0.2 M PCA (see above), the acid-insoluble fraction was heated in 4 ml. 0.4 M PCA at 100° C. for 30 minutes to hydrolyze nucleic acids.[8] The residue after centrifugation was washed once with 4 ml. 0.4M PCA and then dissolved in 0.5 ml. concentrated formic acid; 0.2 ml. was plated on an aluminum planchet for measurement of radioactivity in a flow counter.

Presentation of data. For direct comparison of all experiments, specific activity values have been calculated for a precursor specific activity of 2 by 10^6 c.p.m. per micromole. Adenine-8-C^{14} of 1 to 2 by 10^6 c.p.m. per micromole, glycine-2-C^{14} of 2 by 10^6 c.p.m. per micromole and thymidine-H^3 of 0.5 by 10^6 c.p.m. per micromole were used.

Table I. Incorporation of adenine-8-C^{14} into human leukocyte suspensions

			c.p.m./μM			
Patient	*Disease*	*Minutes*	*RNA Adenine*	*DNA Adenine*	*RNA Guanine*	*DNA Guanine*
A. W.	Normal	60	1,180	B.L.*	117	B.L.
		120	974	B.L.	206	B.L.
M. S.	Granulocytosis	60	2,590	B.L.	135	B.L.
A. N.	CLL	60	58,200	B.L.	—	—
W. H.	CLL	60	17,400	609	531	B.L.
		120	25,200	467	316	B.L.
B. L.	CLL	120	24,400	126	947	111
J. M.	CGL	60	9,480	213	2,040	68
D. C.	CGL	120	16,700	506	951	83
C. Fr.	CGL	60	13,300	556	402	280
		120	21,400	1,220	1,370	444
C. L.	CGL	60	25,200	825	1,030	54
C. Fo.	CGL	60	17,000	992	1,770	116
R. H.	CGL	60	34,400	1,030	1,370	49
A. M.	CGL	60	26,600	2,860	2,010	350

*B.L. = background level.

Results

Table I gives values for incorporation of adenine-8-C^{14} into nucleic acid components by suspensions from leukemic patients before treatment, a normal subject, and a patient with a high granulocyte count of unknown etiology (total W.B.C. = 69,000; 81 per cent mature neutrophils). There was no significant incorporation into DNA by the normal subject or by the patient with a high count of mature neutrophils, and incorporation into RNA was much lower than it was with leukemic suspensions. With the 3 chronic lymphocytic leukemia (CLL) suspensions, the radioactivity of DNA adenine was in the lower range of chronic granulocytic leukemia (CGL) values at 60 minutes, but at 120 minutes this labeling (along with that for RNA guanine) had not increased. As is shown in Tables I (C. Fr.) and II, specific activities of DNA adenine with CGL suspensions increased almost linearly between 1 and 2 hours. The low nonlinear labeling with the CLL suspension is perhaps a base exchange or "renewal" reaction not involving actual synthesis of DNA.

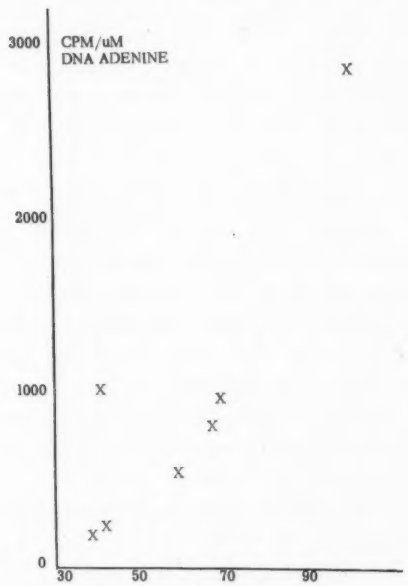

Fig. 1. Weighted percentages of immature cells versus specific activity of DNA adenine with leukocyte suspensions from chronic granulocytic leukemia.

The incorporations into CGL blood in 60 minutes have been arranged in order of values for DNA adenine. Weighted percentages of immature cells were calculated for the 7 CGL patients from the actual percentages as follows: metamyelocytes were given a weight of 1.0, myelocytes 1.5, promyelocytes 2.0, and blast cells 2.5. When the total weighted percentages are plotted against the specific activities of DNA adenine (Fig. 1), only the value for Patient R. H. falls out of line. However, in this short series of CGL patients, all with high total leukocyte counts and significant numbers of immature cells, it would be premature to make any definite statement of correlation between

certain types of immature cells and the degree of incorporation into DNA. Incorporation into RNA adenine and guanine, on the other hand, did not seem to depend on the immaturity of the cells.

Using thymidine-H^3 and stripping film autoradiography, Bond and associates[9] found that this DNA precursor was incorporated well into the blood cells of patients with acute or chronic myelocytic leukemia, but not into the small typical leukemia cell of CLL. There were only small numbers of labeled cells in normal blood. Wells and Winzler[10] found that formate was incorporated into DNA thymine by CGL suspensions, but not to any extent by CLL suspensions. Since our results with a few nonleukemic and CLL subjects also showed low incorporation into DNA, the remainder of the work to be reported was done with CGL suspensions.

Table II shows comparison of the incorporation of adenine by a CGL suspension under aerobic and anaerobic conditions for 1, 2, and 4 hours. Net gas evolution was higher under aerobic conditions, as were specific activities of RNA adenine and guanine. However, although labeling of DNA adenine and guanine was about the same aerobically and anaerobically at 1 hour, values were higher anaerobically at 2 and 4 hours. It was felt that the aerobic conditions better approximated those of the blood, thus subsequent experiments (most of which were for 1 hour) were carried out under 95 per cent air plus 5 per cent CO_2.

The rate of increase in labeling was greatest during the first hour for RNA adenine, and during the second hour for RNA guanine. Aerobically, the incorporation into DNA adenine was nearly linear for the 4 hour period. Incorporation into DNA guanine was low but measurable at 1 hour, and continued to increase with perhaps a somewhat higher rate. Incorporation into all components for 1 and 2 hours was essentially the same with 0.5, 1.0, and 2.0 μM adenine per flask (unpublished data).

Table III shows the effect of 4 normal sera on incorporation by 4 cell suspensions. Results are expressed as the ratio of specific activities from duplicate

Table II. Incorporation of 2.0 μM adenine-8-C^{14} into CGL leukocytes under aerobic and anaerobic conditions

Suspension, Patient R. H.	*Net μl evolution*	*c.p.m./μM.*			
		RNA adenine	*DNA adenine*	*RNA guanine*	*DNA guanine*
95% Air + 5% CO_2.					
1 hour	1,567 ± 73*	48,440 ± 2,060	877 ± 16	1,082 ± 39	42 ± 2
2 hours	2,124 ± 24	76,720 ± 2,110	1,869 ± 36	4,839 ± 117	216 ± 13
4 hours	2,973 ± 22	83,080 ± 2,790	3,806 ± 45	7,050 ± 380	711 ± 9
95% N_2 + 5% CO_2.					
1 hour	1,088	42,110 ± 1,710	845 ± 133	1,027 ± 9	37
2 hours	1,686 ± 79	67,510 ± 2,010	2,704 ± 149	4,035 ± 232	327 ± 13
4 hours	2,179 ± 206	65,010 ± 919	5,275 ± 205	5,145 ± 85	848 ± 98

*Variation from the mean of duplicate flasks.

Table III. Effect of serum on incorporation into CGL leukocytes

$$\frac{\text{Specific activity with serum}}{\text{Specific activity without serum}}$$

	Experiment 1 25% serum S.	*Experiment 2* 25% serum S.	*Experiment 3* 25% serum C.	*Experiment 4* 100% serum R. W.
Adenine-8-C¹⁴.				
RNA adenine	1.17	1.31	1.32	1.30
DNA adenine	.64	.87	.51	.52
RNA guanine	.76	.44	.99	—
DNA guanine	.47	.28	—	—
Thymidine-H³.		*25% serum A. W.*		
DNA	—	.73	.72	.39

Table IV. Incorporation of 1.0 μM pyrimidine compounds compared to adenine-8-C¹⁴

Patient C. Fo., precursor	*Hours*	*c.p.m./μM*			
		RNA cytosine	*DNA cytosine*	*Uracil*	*Thymine*
Cytosine-2-C¹⁴	1	4,174 ± 354*	250 ± 34	608 ± 10	230 ± 74
	2	4,504 ± 142	314 ± 78	3,144 ± 50	412 ± 10
Cytidine-2-C¹⁴	1	39,440 ± 2,990	824 ± 32	30,760 ± 650	544 ± 10
	2	41,360 ± 3,150	1,784 ± 110	44,480 ± 430	2,224 ± 54
		RNA adenine	*DNA adenine*	*RNA guanine*	*DNA guanine*
Adenine-8-C¹⁴	1	17,040	992	1,774	116

*Variation from the mean of duplicate flasks.

flasks containing 25 or 100 per cent serum to specific activities from duplicate flasks containing the glucose-salts medium. The sera had been dialyzed for two 2 hour periods at 3° C. against two 100 times volume portions of the glucose-salts medium. Compared to control without serum, incorporation of adenine into RNA adenine was higher, into RNA guanine lower or unchanged, and into DNA adenine and guanine lower. Incorporation of thymidine-H³ into DNA was also lower. Thus serum was not used in the assay system, since specific activity values for DNA were lower and magnitude of effects on all nucleic acid components was variable. To test the possibility that protein material in the reaction mixture might be stimulatory, 1 per cent crystalline beef albumin was added to flasks for 1 hour incorporations of adenine and thymidine. However, this addition resulted in lower specific activities for both RNA and DNA bases.

In another publication,[1] we shall describe the incorporation of purine and pyrimidine bases, nucleosides, and nucleotides by CGL suspensions. Adenine-8-C¹⁴ was the purine precursor chosen for more detailed study of assay conditions, since it is well incorporated into both RNA and DNA and is readily obtainable. However, for some tests it is also necessary to have a pyrimidine precursor, and, except for cytosine, the pyrimidine bases are poorly

Table V. Incorporation into CGL leukocytes before and after x-radiation therapy

X-radiation	Leukocytes ($\times 10^3$/mm.^{3})	Adenine-8-C^{14} RNA adenine (c.p.m./μM)	Adenine-8-C^{14} DNA adenine (c.p.m./μM)	Adenine-8-C^{14} RNA guanine (c.p.m./μM)	Adenine-8-C^{14} DNA guanine (c.p.m./μM)	Glycine-2-C^{14} protein (c.p.m./mg.)	Thymidine-H^3 DNA (c.p.m./mg.)
Experiment 1. Patient A. M., 1 hour incubation.							
Before:							
4 days	412	26,620	2,862	2,011	350	1,774 ± 72	2,760 ± 100
3 days	412	29,390 ± 2,470*	3,026 ± 287	2,626 ± 7	—	1,658 ± 32	3,100 ± 80
1 day	—	27,810 ± 10	3,092 ± 229	2,333 ± 158	402	—	3,120 ± 140
6 hours	419	38,800 ± 270	3,524 ± 67	2,350 ± 238	539 ± 32	1,642 ± 88	2,640 ± 80
After:							
6 hours	405	33,010 ± 1,960	2,522 ± 164	1,702 ± 131	374	1,618 ± 98	2,440 ± 40
24 hours	485	35,100 ± 2,970	3,146 ± 310	1,718 ± 160	440 ± 19	1,348 ± 48	2,280 ± 100
7 days	164	20,900 ± 1,000	719 ± 67	1,231 ± 31	78 ± 9	1,090 ± 78	980 ± 40
Experiment 2. Patient C. Fr., 2 hour incubation.							
Before:							
1 day	178	21,380 ± 1,000	1,218 ± 78	1,368 ± 30	444 ± 46	291 ± 22	
After:							
1 day	198	22,100 ± 1,880	1,326 ± 82	1,498 ± 40	484 ± 70	307 ± 19	
2 days	101	22,840 ± 400	636 ± 42	1,512 ± 52	208 ± 28	282 ± 2	
5 days	41	14,520 ± 630	240	1,474 ± 126	118	138 ± 10	

*Variation from the mean of duplicate flasks.

incorporated. The incorporation of cytosine, cytidine, and adenine by the same suspension is compared in Table IV. Since specific activities with cytosine are lower than those with adenine, we have used the nucleoside, cytidine-2-C^{14}, in our assay system. Under the conditions described for adenine, cytidine is well incorporated into both RNA and DNA.

In addition to studying the effects of chemotherapeutic agents on leukocytes in vitro, we wished to determine if the assay system could be used for detecting changes which might be measured in vitro following in vivo therapy with radiation or drugs. In was thus necessary to investigate the variation in incorporation by suspensions of leukocytes drawn from a patient during several days before treatment. Results are given in Experiment 1 of Table V, and show that, with careful standardization of methods, reasonably good replication over a period of several days can be expected. Patient A. M. was treated with x-radiation each day for 6 days. Blood was withdrawn at 6 and 24 hours after start of treatment to determine if x-radiation affected the in vitro incorporation of the white cells before marked changes occurred in the total count and in the differential, which are given in Table VI. Total count and percentage of immature cells began decreasing gradually after the fourth posttreatment day, but the differential did not change greatly until the seventh day, when the percentage of blast cells had dropped from 24 per cent on the previous day to 8 per cent. No marked change over pretreatment

Table VI. Differentials of patients used for x-radiation studies

	*Patient A. M.**			*Patient C. Fr.†*			
	Before 3 days	*After 24 hours*	*7 days*	*Before 1 day*	*After 1 day*	*2 days*	*5 days*
Total leukocytes ($\times 10^3$/mm.^{3})	412	485	164	178	198	101	41
Neutrophils	32.8	30.6	41.6	50.2	56.2	59.8	81.4
Eosinophils	2.0	1.4	1.0	1.2	1.4	1.4	2.0
Basophils	0.4	0	0.2	0	0	0	0
Monocytes	4.2	6.2	11.2	0.4	0	0.2	5.2
Lymphocytes	3.8	1.0	6.0	1.4	1.6	1.2	1.8
Young or atypical lymphocytes	0	0	0	0	0.2	0.2	0
Metamyelocytes	6.6	5.8	9.0	20.0	9.6	12.0	7.4
Myelocytes	22.8	17.6	19.0	17.4	25.0	21.6	1.0
Promyelocytes	0.8	0.8	0.4	1.0	0.2	0	1.0
Blast cells	23.6	32.6	8.0	6.4	5.4	3.0	0.2
Normoblasts	0.6	1.4	0.2	0	0	0.4	0
Pathologic and unclassified	3.4	2.6	3.4	0.8	0.4	0.2	0
% Above line	43.2	39.2	60.2	53.2	59.4	62.8	85.2
% Below line	56.8	60.8	39.8	45.6	40.6	37.2	14.8

*Patient A. M., a 64-year-old man, received x-radiation to the spleen on 6/30, 7/1, 7/2, 7/3, 7/5, and 7/7.

†Patient C. Fr., a 32-year-old woman, received x-radiation to the spleen on 5/27, 5/28, 5/29, and 6/1.

values for the incorporation of adenine occurred except in the 7 day sample; RNA adenine and guanine were lower, but the largest changes were in DNA adenine and guanine, which were only 23 and 18 per cent of corresponding values for the 24 hour sample. The incorporation of thymidine-H^3 into DNA was also determined as a measurement of DNA labeling without concomitant labeling of RNA. As with the incorporation of adenine into DNA bases, there was no marked change until the significantly lower radioactivity of the 7 day sample. Incorporation of glycine into protein decreased somewhat at 24 hours, with a definitely lower specific activity at 7 days.

In Experiment 2 of Table V, a patient receiving x-radiation was studied until the ratio of mature to immature cells was greater than that for the 7 day sample of Experiment 1. The incorporation of glycine into protein and adenine into RNA adenine did not change significantly until the 5 day sample; values for RNA guanine were about the same for all samples. On the second day after treatment, specific activities of DNA adenine and guanine had decreased to 48 and 43 per cent of the values for the first posttreatment day and, on the fifth day, to 18 and 24 per cent of the first day values. Although there was not such a marked change in the differential between the first and second days (Table VI), the total count decreased by about 50 per cent.

These results demonstrate that blood leukocytes from patients being treated with x-radiation do not change greatly in the ability to incorporate the above precursors until a significant change in total count and differential has occurred.

Discussion

Various other workers have studied the in vitro incorporation of nucleic acid and protein precursors into human leukocytes. Hamilton[11] incubated leukocytes from patients with acute myelogenous leukemia with C^{14}-labeled adenine, guanine, and 2, 6-diaminopurine for 4 hours in homologous plasma. Scott and associates[12] studied the incorporation of C^{14}-labeled adenine, glycine, and formate into DNA and RNA bases of normal and leukemic leukocytes during 4 and 8 hour incubations in 50 per cent normal serum–Hanks' solution. They found no effect of 6-mercaptopurine or its riboside on the incorporation of adenine or glycine, but an inhibition of formate incorporation by amethopterin.

Winzler and colleagues[10, 13, 14] have measured the incorporation of C^{14}-labeled formate, glycine, adenine, orotic acid, uracil, and thymine into RNA and DNA bases of normal and leukemic cells suspended in glucose-supplemented normal serum. They have particularly studied the incorporation of formate and its inhibition by amethopterin.[10] Winzler and associates[15] also measured the effect of a number of drugs on the incorporation of formate-C^{14} and glycine-1-C^{14} into the gross protein plus nucleic acids fraction of leukocytes, and concluded that this system did not lend itself to the screening of potential chemotherapeutic agents. No correlation could be made between clinical and in vitro effects of chemotherapeutic agents used in the treatment of leukemia, and most of the compounds investigated had little or no effect on incorporation of either formate or glycine into the gross cell fraction isolated.

We have not been interested primarily in a quick screening method for potential antileukemic drugs, but rather in devising a system to investigate effects of chemotherapeutic agents on various parameters of nucleic acid metabolism. Most of our studies have been with a chemically defined medium for 1 or 2 hour periods, during which time measurable incorporation into RNA and DNA occurred without gross degradation of acid-soluble compounds by catabolic reactions,[1] and without the necessity of sterility control. The medium is more reproducible than one requiring serum, and the system can also be used for investigating normal and pathologic sera. We are particularly interested in testing compounds, such as alkylating agents, which are not obvious antipurine or antipyrimidine drugs. Information obtained, it is hoped, can be used in studies on both the mechanism of drug action and the resistance of leukemic cells to these agents.

We wish to express our appreciation to Elaine Joranger for technical assistance.

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A new hemoglobinopathy of unusual genetic significance

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A 47-year-old Negro man is described whose hemolysate contains 4 major hemoglobin components. By starch and agar electrophoresis, recombination experiments, and "fingerprinting" technique, these components are (1) normal hemoglobin A_1, (2) a new variety of hemoglobin D (alpha chain abnormal), (3) hemoglobin C (beta chain abnormal), and (4) a hybrid of hemoglobins D and C (alpha and beta chains abnormal). The subject had a mild, slightly uncompensated hemolytic anemia to which no symptoms were attributable. The biochemical data concerning his hemoglobin are compatible with the hypothesis that synthesis of alpha and beta chains is under the control of at least 2 separate genes, but the combination of these chains to form the whole globin portion of the hemoglobin molecule is governed more by biochemical than genetic laws.

Since the introduction of electrophoretic methods for the differentiation of sickle from normal hemoglobin,[1] a wide variety of human hemoglobins has been described. Sickle hemoglobin is now known to differ from normal hemoglobin by the substitution of a single amino acid residue (valine for the normal glutamic acid) in the globin portion of the sickle hemoglobin molecule.[2] The globin portion of the normal hemoglobin molecule is composed of 2 types of polypeptide chains termed alpha and beta. There are 2 alpha and 2 beta chains in hemoglobin A_1.[3] Thus, by chemical shorthand, this hemoglobin may be written

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$\alpha_2\beta_2$ (the subscript "2" has the same significance as it does in H_2O). It has been proposed that the synthesis of each kind of chain is under separate genetic control but that their combination after synthesis to form the final hemoglobin molecule may be a function of biochemical rather than genetic processes.[4]

The elegant work of several investigators, notably Itano and associates[4] and Hunt and Ingram,[5] has demonstrated that the amino acid substitutions responsible for the abnormal hemoglobins S and C reside in the beta chains while the alpha chains are normal. In the abnormal hemoglobins I and Hopkins-2, the alpha chains are abnormal while the beta chains are normal. Hence, abnormal hemoglobins can result from mutation of genes controlling the synthesis of either the alpha or the beta chain.

According to this concept it should be possible for an individual to inherit simultaneously a gene for an alpha as well as a beta chain abnormality. Such a person would form 4 hemoglobins, one completely normal, one containing abnormal beta chains along with normal alpha chains, one containing abnormal alpha chains and normal beta chains, and one with both chains abnormal. The discovery of a patient with 4 major hemoglobin components and the elucidation of the structural abnormalities in the molecular species of these components form the substance of this report. The findings in this patient support these recent concepts concerning the mechanism of hemoglobin synthesis and structure.

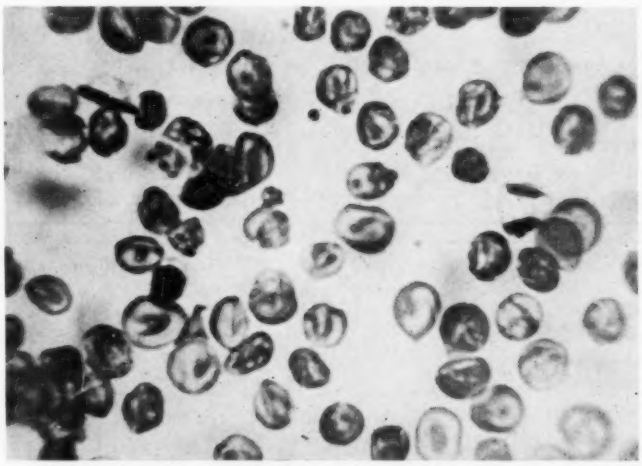

Fig. 1. Peripheral blood smear from Patient J. E. (Wright's stain. Original magnification ×930).

Case report

Patient J. E. (DCGH No. 62448), a 47-year-old Negro man, was admitted to District of Columbia General Hospital complaining of abdominal pain. He had been injured in an accident 2 years previously. At another hospital, a ruptured spleen was removed and eventration of the diaphragm repaired. The spleen weighed 413 grams which included a large hematoma. Microscopic sections* contained no specific lesions. The pathologic diagnosis was splenomegaly of undetermined origin with acute rupture secondary to trauma. The

*We are grateful to Dr. R. F. McGuire, Pathologist, Prince Georges County Hospital, Md., for these data.

patient also sustained a fracture of the left leg. Chronic osteomyelitis, resistant to antibiotic and conservative treatment, developed subsequently at the fracture site. The past and family histories were noncontributory.

There were few positive findings during physical examination at the time of admission. A laparotomy scar and the draining sinuses over the left thigh were present. The left kidney was abnormally motile. Clinical and laboratory evaluation, including x-rays of the gastrointestinal tract, gall bladder, and kidneys, was normal except for ptosis of the left kidney and a redundant, rather atonic, colon. Mild abdominal discomfort was largely alleviated by therapy with Senoket.* The hematocrit was 38 per cent and the white cell count and differential normal. Red cell morphology was strikingly abnormal (Fig. 1). There were target and folded cells, microspherocytes, and cells containing elongated hemoglobin crystals. Because of this abnormal morphology, further studies were undertaken.

Table I

Procedure	*Normal*	*Patient J. E.*	*Comment*
Hematocrit	47±5	38	
Mean corpuscular volume	87±5	72	
Mean corpuscular hemoglobin concentration	32–36	32	
Reticulocyte count (per cent)	0.5–1.5	1.8	
Siderocytes (peripheral blood)*	2%	25–30%	
Serum iron	80–140	123	
Unsaturated iron-binding capacity		140	
Per cent saturation of iron-binding protein	30%	47%	
Plasma T½ Fe^{59}	60-150	53	
Plasma iron turnover (mg./Kg./day)	0.45±0.11	1.03	2× normal
Red cell Fe^{59} uptake	80–100%	74%	
Red cell mass (ml./Kg.)	28–30	26	
Observed T½ Cr^{51}-tagged red cells (days)	28–32	16	
Corrected T½ Cr^{51} (days)[6]		24.5	
Mean cell life (days)[6]		35	1/3 normal

*We are grateful to Dr. Neal Barrett, Walter Reed Army Medical Center, Washington, D. C., for these studies.

Methods

Routine hematologic observations were done by standard methods.[6] The technique of Huff[7] for measuring plasma iron turnover was modified by injecting the tracer dose of Fe^{59} citrate (Ferrutope†) directly into the blood stream. Four serum samples were obtained 20 to 60 minutes later and 2 ml. aliquots counted in a well-type scintillation counter. Counts per minute per milliliter were plotted against time on semilogarithmic graph paper and a straight line was fitted to the points. The turnover rate was calculated in milligrams per kilogram per day with the use of the dilution volume obtained by extrapolating the disappearance curve to zero time. Serum iron was measured with bathophenanthroline as the color reagent.[8] Three hours after the tracer dose of Fe^{59}, 250 mg. iron-dextran (Imferon)‡ was given intramuscularly to "block" recirculation of iron from extramedullary depots back to erythropoiesis.[9]

Paper electrophoresis was done at pH 8.6 by a method slightly modified from Singer.[10] Agar electrophoresis was done at pH 6.5[11] and starch block electrophoresis at pH 8.6.[12] Alkali denaturization was used to determine hemoglobin F.[10] A sickling test was performed with sodium metabisulfite as reducing agent.[13]

*Senoket (senna), The Purdue Frederick Company, New York, N. Y.
†E. R. Squibb & Sons, New York, N. Y.
‡Imferon (iron-dextran), Lakeside Laboratories, Inc., Milwaukee, Wis.

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Recombination studies were done by means of a modification of the technique outlined by Itano and Singer.[14] The patient's purified hemolysate was converted to cyanmethemoglobin and, by means of starch block electrophoresis, the various fractions were separated and eluted with distilled water. Aliquots of the fractions were mixed and divided into control and reaction portions. The latter was dialyzed against sodium acetate buffer at pH 4.5, ionic strength 0.02, for 4 hours at 5° C. A precipitate of denatured proteins was removed by high speed centrifugation. The control and reaction mixtures were concentrated by evaporation and again separated by starch electrophoresis.

"Fingerprinting" of the purified fractions was carried out by a modification of Ingram's procedure,[15] as were the tryptic digests. The hemoglobin fractions were isolated as the cyanmethemoglobins by starch block electrophoresis at pH 8.6 and eluted with buffer of the same pH. The hemoglobin was precipitated by the addition of zinc acetate solution (final concentration of zinc, 20 mEq. per liter) and the precipitate then dissolved in a solution of disodium ethylenediaminetetraacetate which had been previously adjusted to pH 8.6 with sodium hydroxide. For ease of comparison, the tryptic digest of the several fractions were also separated in parallel on a single sheet of paper with the use of a cooled plate, high voltage paper electrophoresis apparatus, and a pyridine-acetic acid buffer of pH 6.5.

Results

Clinical studies. Routine hematologic and erythrokinetic studies are summarized in Table I. The hematocrit and red cell mass are slightly less than normal. The red cell survival is shortened and there is slightly reduced red cell Fe^{59} incorporation. Since a normal marrow should readily compensate for the degree of hemolysis found, the mild anemia is a result of relative bone marrow failure.

As determined by starch block electrophoretic analysis, the patient's hemoglobin separated into 4 separate components (Fig. 2). One had the mobility of A_1 (or F) and represented 18 per cent of the total. The alkali-resistant fraction constituted 6.5 per cent of the hemoglobin present. The component in the S or D position comprised 14 per cent of the total. Since it did not separate from A_1 on agar (Fig. 3), and there was no sickling, it had the characteristics of hemoglobin D, but will be designated "D" for reasons described

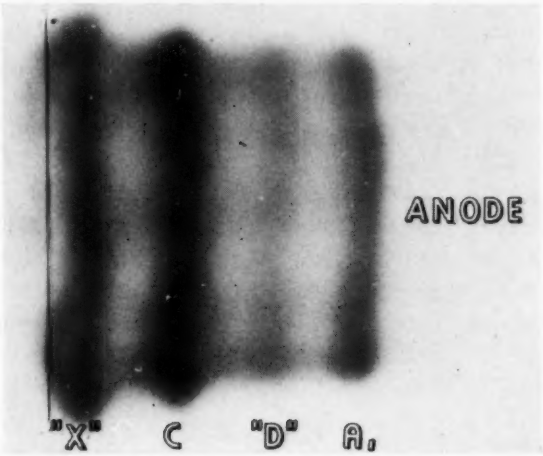

Fig 2. Starch block electrophoresis of the hemolysate (as cyanmethemoglobin) in barbital buffer (0.05 molar, pH 8.6). The narrow black line represents the origin.

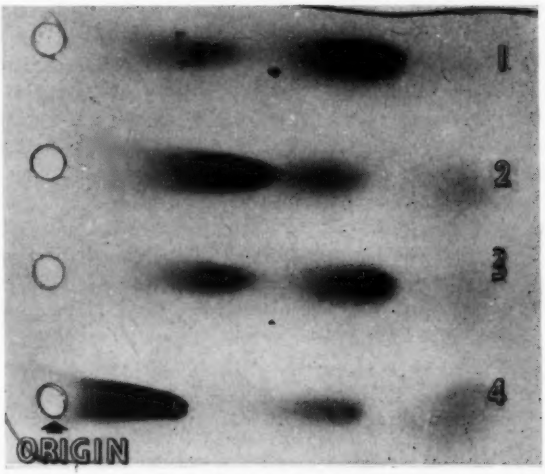

Fig 3. Agar electrophoresis of hemolysates (as cyanmethemoglobin). Citrate buffer, pH 6.5. *(1)* AS (sickle cell trait), *(2)* SD (sickle cell–hemoglobin D disease), *(3)* AS, *(4)* Patient J. E. Origin as marked at left.

below. Hemoglobin C constituted 44 per cent of the total. Finally, 24 per cent of the total hemoglobin had the unique property of a slower electrophoretic mobility than hemoglobin C at pH 8.6. This new hemoglobin will temporarily be referred to as hemoglobin "X."

Blood samples from the subject's wife and son were examined but paternity was excluded by blood grouping tests.* No other members of his family could be located.

"Fingerprinting" of the hemoglobin components. Tryptic digests of the several hemoglobin components were studied initially by one-dimensional high voltage paper electrophoresis (HVPE) (Fig. 4). By this technique, fraction "D" was found to contain 2 peptide spots not present in the pattern of fraction A_1. The tryptic digest of the hemoglobin C fraction likewise possessed a peptide spot not found with either fraction "D" or A_1. A second new peptide present in the digest of hemoglobin C[16] was not visible in the strip prepared by HVPE as it remained in the neutral band region. Both new peptides were visible on the "fingerprints." Finally, digest of fraction "X" was demonstrated to possess all 3 abnormal spots identified in fractions C and "D."

These findings were corroborated by "fingerprinting" the individual tryptic digests (HVPE at pH 6.5 followed by paper chromatography using a butanol–acetic acid–water solvent mixture). The fraction identified as A_1 gave a "fingerprint" indistinguishable from that obtained with digests of whole hemoglobin from normal persons. The hemoglobin C fraction gave a "fingerprint" identical to the pattern previously described for that hemoglobin.[16] The hemoglobin "D" "fingerprint" showed the 2 new spots first located by one dimensional HVPE. (One of the new spots was superimposed upon a

*We are grateful to Dr. Paul Schmidt, National Institutes of Health, Bethesda, Md., for these studies.

"normal spot" but could be distinguished therefrom by the marked increase in color reaction with Ninhydrin.*) The "X" fraction showed the combination of the changes described for C and "D."

"Hybridization" of the hemoglobin fractions. Fig. 5 represents the recombination experiments with the various hemoglobin components. At the top (*1*) is the control mixture of the C and "D" fractions. After acid dissociation

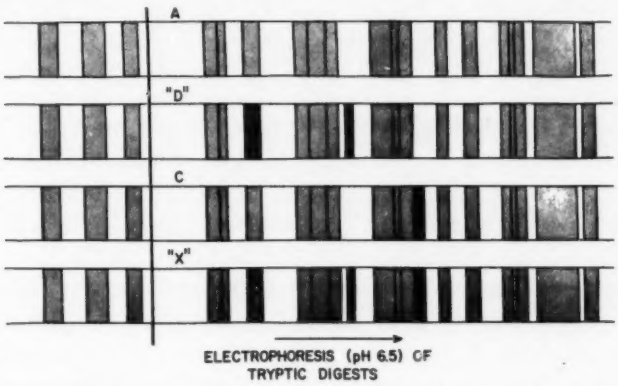

Fig. 4. Schematic representation of the one-dimensional electrophoretic patterns obtained from tryptic digests of the isolated hemoglobin fractions. The "new" peptide spots present in the abnormal hemoglobin fractions and not present in the hemoglobin A fraction are indicated by the solid black areas. The origin is indicated by the continuous vertical line.

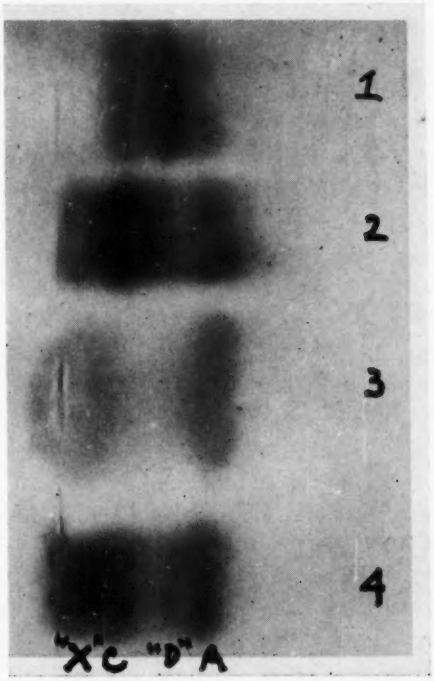

Fig. 5. Results of recombination experiments. Starch block electrophoresis, veronal buffer pH 8.6, ionic strength 0.05, 50 Ma. for 16 hours. See text.

*Ninhydrin (1, 2, 3-Indantrione hydrate), Merck Sharp & Dohme, Inc., Philadelphia, Pa.

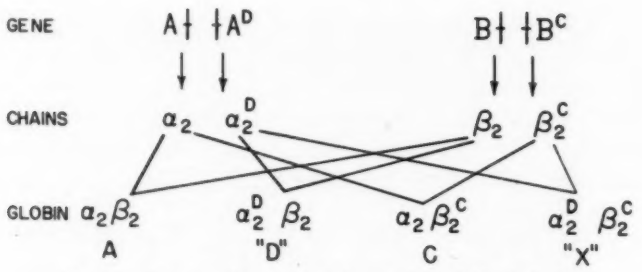

Fig. 6. Proposed genetic schema for Patient J. E.

and recombination (*2*) hemoglobin "X" and A_1 are present. Next (*3*) is the control mixture of C and A_1. After acid dissociation and recombination (*4*) "X" and probably "D" are present.

Discussion

From a clinical viewpoint, this case has interest largely as a curiosity to be distinguished from other mild thalassemia-like anemias and causes for splenomegaly. The first clue that there was something unusual about the subject's hemoglobin came from the study of the smear of his peripheral blood. The red cell morphology was quite striking, not dissimilar from that found in hemoglobin C–thalassemia disease.[17] The intraerythrocyte crystals which were seen have been described previously in homozygous hemoglobin C disease and have been noted to be particularly prominent after splenectomy.[18, 19] They are to be clearly distinguished from sickled forms.

The erythrokinetic studies indicate that a mild uncompensated hemolytic anemia was present. The role of chronic infection in the genesis of this relative marrow failure cannot be assessed fully since preinfection hematocrit values are not known. As in many patients with abnormal hemoglobin syndromes, the degree of anemia may be dependent more upon relative marrow failure than upon the rate of hemolysis.[17]

The major interest in this patient centers about the biochemical studies and their implications. Analysis by starch electrophoresis revealed the presence of 4 different hemoglobins. The "fingerprinting" technique of hemoglobin analysis indicated that the A_1 fraction was indistinguishable from the hemoglobin A_1 of normal individuals and that the fraction labeled C was biochemically identical to previously described samples of hemoglobin C. The hemoglobin "D," on the other hand, was found to be a new hemoglobin unlike any previously described.*

The in vitro formation of an "X" fraction by "hybridizing" a mixture of the C and "D" indicates that the abnormal chains in the 2 molecules are capable of combining with each other. Others have suggested that such in vitro recombination is evidence that the abnormal chains found in patients are of different kinds, i.e., one is an abnormal alpha and the other an abnormal beta chain.[4] Similarly, the formation of hemoglobin A_1 during this hybridization

*A hemoglobin possibly identical to this is currently under investigation in V. M. Ingram's laboratory.[20]

experiment is evidence that there are normal chains in the 2 initial hemoglobin fractions (C and "D") and that they too are of different kinds. Since the abnormality in hemoglobin C is known to be located on the beta chain, it follows that the hemoglobin "D" lesion is present on the alpha chain.*

The localization of the chemical defect of the hemoglobin "D" in the alpha chain in this patient is also suggested by the "fingerprinting" studies of this fraction. In addition to the 2 new spots seen in this "fingerprint," one normally present peptide spot (designated as No. 3 by Ingram) was missing. This peptide is known to come from the alpha chain[21] and hence the hemoglobin "D" abnormality may be presumed to lie in this chain.

According to the hybridization concept just expressed the occurrence of 4 hemoglobins in the patient's original hemolysate may be explained by assuming that he produces 4 different hemoglobin chains—α, $\alpha^{\text{"D"}}$, β, and β^{C}. These then combine after synthesis to form 4 distinct hemoglobins, i.e., $\alpha_2\beta_2$ (hemoglobin A_1), $\alpha_2{}^{\text{"D"}}\beta_2$ (hemoglobin "D"), $\alpha_2\beta_2{}^{C}$ (hemoglobin C) and $\alpha_2{}^{\text{"D"}}\beta_2{}^{C}$ (hemoglobin "X") (Fig. 6).

Some support for the above interpretations has been advanced in a recent report[22] describing multiple hemoglobins in an apparently unrelated family. Several members of this family possessed hemoglobin which on electrophoretic analysis resolved into 4 major components having mobilities apparently indistinguishable from those reported herein. The hemoglobins in this family have the same "fingerprint" patterns as we have found for Patient J. E.,[20] but the relative quantity of each hemoglobin was different. The component with next to greatest mobility has been labeled "D" rather than the G designation of Atwater and associates[22] because of the results of agar electrophoresis. Hemoglobins S, D, and G are not readily separable by paper electrophoresis,[23] but may be distinguished by means of agar electrophoresis.[11]

Electrophoretic techniques have played a dominant role in the discovery and designation of new hemoglobins. The addition of biochemical methods has added much to our knowledge of the molecular structure of hemoglobin. By combining electrophoresis, tryptic digestion, chromatography, and in vitro hybridization in the study of the hemoglobin of our patient, it has been found that hemoglobin "D" has a previously undescribed structure, information not available from electrophoretic studies alone. In accord with recent decisions about nomenclature,[25] this hemoglobin should be designated $D\alpha_{\text{Washington}}$. The other "new" hemoglobin, heretofore designated "X," appears to be a naturally occurring hybrid of hemoglobins "D" and C. According to a recent conference on nomenclature[25] this hemoglobin might be termed "hemoglobin Washington" (or Washington-1), but its identity as a hybrid more descriptively suggests the name "Hemoglobin $DC_{\text{Washington}}$."

The technical assistance of Mona M. Gieschen, M.T., ASCP, and Kenneth N. Vertrees, HMC, USN, is gratefully acknowledged.

Addendum: Since this report was prepared another patient possessing 4 hemoglobins has been reported.[24]

*The precise amino acid sequence alteration occurring in this hemoglobin is currently being investigated.

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Creatine and creatinine excretion in leukemia

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When the creatinine excretion of leukemic patients was found too variable to be used as an index of the completeness of 24 hour urine collections, a study was made of its excretion and was correlated with the type of leukemia and the phase of disease. The work was extended to include creatine excretion. A total of 15 patients was studied for periods of 2 to 20 months. In all cases, the creatinine excretion was low, but tended to approach normal levels in periods of remission. Creatinuria was most pronounced in the acute leukemias of children, but it was also significant in leukemic adult men, especially in the active phase in a case of subacute granulocytic leukemia.

The studies of the abnormalities in the metabolic excretory products of leukemic patients have included a number of nitrogenous substances, but very little has been reported on the creatinine and creatine excretions. Waisman and associates[1] found that creatinine, creatine, and the ratio of these 2 substances, excreted by normal children, and by patients with acute leukemia, varied considerably. Sandberg and co-workers,[2] on the other hand, used the creatinine excretion as an index of the completeness of 24-hour collections in patients with different types of leukemia. Our original reason for the determination of creatinine in the urine of leukemic patients was the same as that of Sandberg and co-workers.[2] However, the creatinine excretion was not found constant, neither for different leukemic cases, nor for different periods in the course of the disease in the same individual.

With the finding of variability in creatinine excretion, this study was expanded to include creatine output. The excretory patterns of these substances

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were followed for periods of 2 to 20 months. This report presents the findings on the excretion of these metabolites in various phases of several types of leukemia. The correlation of the data with the clinical and hematologic findings is perhaps of greater significance than the absolute values found.

Methods

Urine. Urine was collected from leukemic patients at various phases of their disease. The 24 hour collections were preserved with thymol, and filtered through a Berkefeld N filter shortly after the end of the collection period to remove cellular debris. Filtered samples that could not be used at once were stored at −10° C.

Analysis. Creatine and creatinine were determined essentially by the method of Clark and Thompson.[3, 4] When the specific gravity of the urine was below 1.015, a 1:5 dilution of urine with water was used, and, when above 1.015, a 1:10 dilution was used. A one milliliter volume of the diluted urine was measured into each of four 25 ml. volumetric flasks. Four milliliters of 1.17 per cent sodium picrate, pH 2.0, was added to each. Two of the flasks were autoclaved at 15 pounds pressure for 30 minutes, then cooled. To each of the 4 flasks, 0.3 ml. 2.5 N NaOH was added, and, after 20 minutes, the volumes were made up to the mark and the density measured at 520 mμ with a Beckman DU spectrophotometer. Standard creatinine solutions were run with each batch of urine samples, and the values obtained for the heated and unheated samples were used to calculate the creatinine and sum of creatinine and creatine, respectively. It is necessary to include standards[4] with each set of determinations, as the readings are sensitive to wavelength setting of the colorimeter, and heating sometimes destroys up to 5 per cent of the creatinine. Albanese and Wangerin[5] claim that 10 per cent may be destroyed.

Materials and subjects

All patients included in this study were admitted to the hospital for verification of the diagnosis of leukemia. Detailed clinical and laboratory tests, including bone marrow aspirations, were performed. The 15 cases of leukemia included in this study are classified in Table I. During the course of illness, frequent bone marrow studies were performed to evaluate the state of disease. The courses were divided into partial or complete remission and relapse, according to the classification of Biesel.[6]

The cases reported received either single or multiple administration of folic acid antagonists, 6-mercaptopurine, adrenocorticosteroid, colcemide, and Myleran. No attempt was made to correlate the urinary creatine and creatinine levels with the specific treatment. The urine samples were collected at random, but one or more samples were procured during different stages of the disease. Due to limitation of space, the history and hematologic course of each case is not given. Three representative cases were selected and their clinical, hematologic, and creatine and creatinine excretions discussed in detail.

Units. Creatinine and creatine excretions have been reported in a number of ways, frequently with the omission of data required for comparing values. The results reported in the present paper are in terms of micromoles excreted per kilogram body weight per hour (micromoles per kilogram per hour). This unit permits the direct addition of creatinine and creatine concentrations without the need of calculating creatine as creatinine. Values used from the references are converted to this unit. The creatinine coefficient (milligrams creatinine per kilogram per day) can be calculated by multiplying the above unit by 2.72. The weight of each patient is given in Table I to permit approximations in other units. The normal values were obtained from 10 urine samples of men, 13 to 48 years of age, and in good health. These values were found to agree with analyses reported in the literature.

Results

The findings on all patients studied are summarized in Fig. 1. Each column of symbols represents an individual patient in the order in which the patients

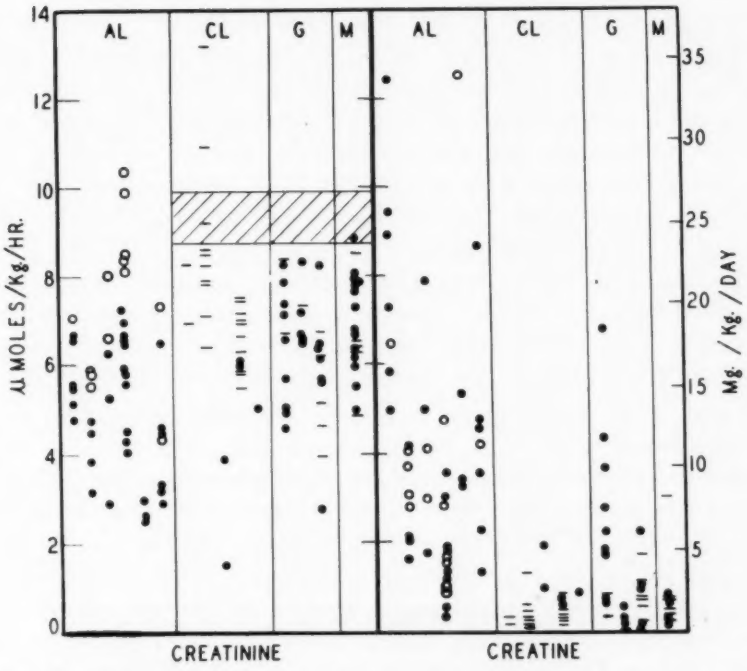


Fig. 1. Creatinine and creatine excretion in leukemic patients. The solid dots represent the active leukemic phase of the disease (relapse), the circles, the disease in complete remission, and the dashes, incomplete remission according to the classification of Biesel.[6] The shaded area in the creatinine graph indicates the average of normal values ± one standard deviation. Normal values for creatine are not shown, as the values depend upon the individual. The columns of symbols from the left to right correspond to the individuals as listed in Table I.

Table I. Summary of leukemic cases studied

Leukemia type	*Case No.*	*Age in years*	*Sex*	*Weight in kilograms**
Acute lymphatic	AL-1	7	F	23—29
	AL-2	2	M	10—11
	AL-3	14	M	52—58
	AL-4	12	F	36—65
	AL-5	1.4	M	10
	AL-7	2.5	F	13—15
Chronic lymphatic	CL-1	65	M	89
	CL-2	47	M	78—80
	CL-3	74	F	65
	CL-4	74	M	72—80
	CL-5	84	F	47
Acute granulocytic	AG-2	54	M	56—50
Chronic granulocytic	CG-1	67	M	64—69
	CG-2	69	M	74—79
Monocytic	M-1	37	M	65—56

*The range indicates the weight at the beginning and the end of each case, respectively.

are listed in Table I. Most of the creatinine excretions are below the range of the normal values in all stages of the disease. In the acute lymphatic leukemia, the creatinine excretions tended to be highest in the remission phase of the disease and lowest in periods of relapse. In these cases a marked creatinuria was present. Creatinuria would be expected, as the patients were children, but, in many, the creatine excretion exceeded that of the creatinine. When correlated with the clinical state, the creatine levels showed a distinct tendency to lower values in the remission phase.

Of the 5 cases of chronic lymphatic leukemia, the first, second, and fourth case had a very steady course. Most of the creatinine values were below the normal, but, because of the steady course, the values could not be correlated with the phase of the disease. The lowest creatinine values in 2 of the cases were recorded just prior to death. On several occasions noticeable amounts of creatine were found.

The granulocytic leukemias are not separated as to type, but the first column in this group is a subacute case which is discussed in more detail (AG-2, in Table I). The other 2 cases are of the chronic type, and the creatinine and creatine excretion patterns are about the same as are found in chronic lymphatic

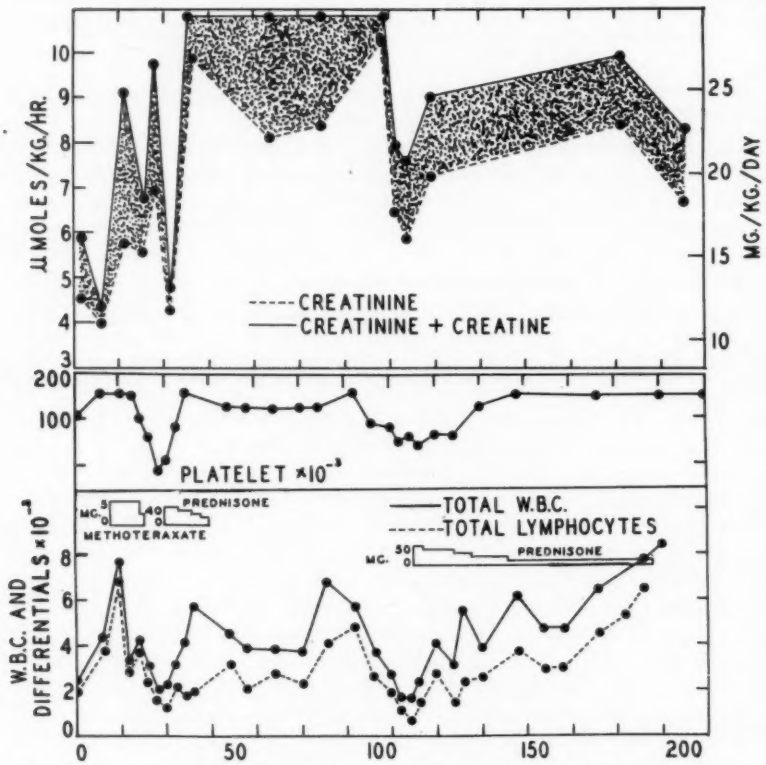


Fig. 2. Relation of creatinine and creatine excretion to the clinical and hematologic findings in a case of acute lymphatic leukemia (AL-4). The amount of creatine excreted is indicated by the shaded area, which is actually the difference between the creatine plus creatinine and the creatinine.

leukemia. Both showed low excretions of creatinine, with creatinuria on several occasions. The case of subacute myelomonocytic leukemia studied showed results which were similar to those of the case of chronic leukemia. Three cases were selected for a more detailed description.

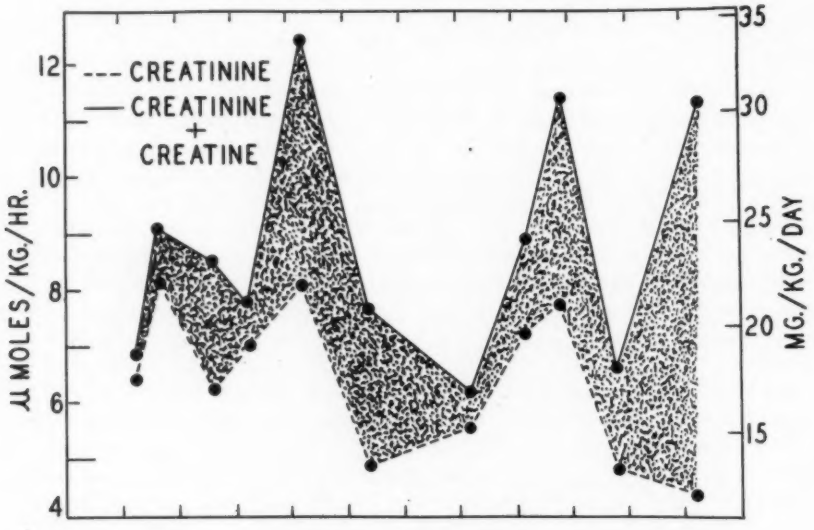
Case reports

Case AL-4. A 12-year-old girl was admitted to the hospital in January, 1958, with a history of a 20 pound weight loss. Clinically she had some cervical lymphadenopathy. The physical examination was otherwise entirely negative. On admission, the hematologic findings were as follows: Hemoglobin, 11 Gm. per cent; R.B.C., 3.5 million per cubic millimeter; platelets, 148,000 per cubic millimeter; reticulocyte, 0.6 per cent; and W.B.C., 2,450 per cubic millimeter. The differential count showed 18 per cent segmented neutrophils and 82 per cent lymphocytes, of which 10 per cent were lymphoblasts. A bone marrow examination revealed a marked depression of erythroid, granulocytic, and megakaryocytic activities. The lymphoblasts constituted 91 per cent of the cell. The entire hematologic course, in relation to treatment and to the creatine and creatinine levels, is shown in Fig. 2. Pretreatment creatinine levels are significantly lower than the average normal values. During this period, the amount of creatinine excreted, in terms of micromoles per kilogram per hour, varied between 4.02 and 6.92. Following a course of antifolic acid and steroid therapy the patient improved hematologically and clinically and remained in a stage of remission for a period of 2 months. In this stage, creatinine excretion was at a normal level, varying between 8.10 to 10.33 μM per kilogram per hour. At the end of this period the patient's condition began to deteriorate. During the relapse state, just prior to the second course of adrenocorticosteroid therapy, there was a sharp decrease in creatinine excretion, the values varying between 5.90 and 6.45 μM per kilogram per hour. Following a second course of steroid therapy, the patient improved, and creatinine values again approached normal levels, ranging from 6.67 to 8.38 μM per kilogram per hour. Creatinuria varied considerably. The amount of creatine excreted during the course of disease varied between 0.36 and 4.76 μM per kilogram per hour.

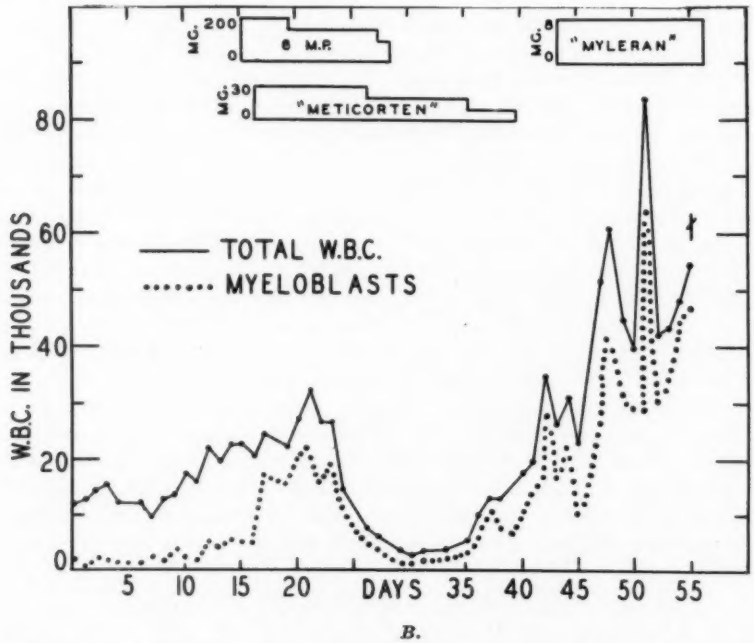
Case AG-2. A 54-year-old white man was admitted to the hospital with a history of backache, arthralgia, and fatigue of 5 months' duration. His physical examination revealed severe pallor and a swollen left knee, which later proved to be an effect of leukemic arthritis. The hematologic findings were as follows: total W.B.C., 12,650 per cubic millimeter; R.B.C., 1.7 million per cubic millimeter; platelets, 29,000 per cubic millimeter; and hemoglobin, 5.75 Gm. per cent. The differential count was: myeloblasts, 11 per cent; promyelocytes, 10 per cent; myelocytes, 9 per cent; juveniles, 14 per cent; stabs, 1 per cent; segmented neutrophils, 7 per cent; eosinophils, 1 per cent; lymphocytes, 40 per cent; and monocytes, 7 per cent. A bone marrow biopsy revealed marked cellularity due chiefly to immature granulocytes. Approximately 24 per cent of the cells were myeloblasts. This case was classified as a subacute granulocytic leukemia. The patient's response to various antileukemic treatments was unsatisfactory. His hematologic course, in relation to treatment and to creatine and creatinine excretions, is shown in Fig. 3. The creatinine excretion followed an interesting pattern in that the amount of excreted creatinine became progressively lower as the leukemic process advanced. The amount of urinary creatinine varied between 4.56 and 8.24 μM per kilogram per hour. Approximately 82 per cent of creatinine values were below the normal range. The patient further showed a very significant creatinuria, the creatine in the urine varying between 0.36 and 6.84 μM per kilogram per hour. At the stage immediately preceding death the creatine exceeded the creatinine levels. Another interesting finding is a suggested correlation between the degree of creatinuria and the hematologic picture, namely, total granulocytes in peripheral blood.

Case CG-2. A 69-year-old white man was first admitted to the hospital in January, 1957, with a swelling of the abdomen and a history of weight loss. Clinically he had considerable hepatosplenomegaly and some lymphadenopathy. The initial hematologic findings were as follows: hemoglobin, 11.9 Gm. per cent; R.B.C., 4.2 million per cubic millimeter; platelets,

128,000 per cubic millimeter; leukocytes, 35,400 per cubic millimeter. The differential count revealed: promyelocyte, 3.5 per cent; myelocyte, 3 per cent; basophils, 2 per cent; and lymphocytes, 7 per cent. The bone marrow findings were compatible with the diagnosis of chronic granulocytic leukemia. The hematologic course, treatments received, and creatine and creatinine excretions are illustrated in Fig. 4. The creatinine excretion was found to vary between 2.76



A.



B.

Fig. 3. Relation of creatinine and creatine excretion to the clinical and hematologic findings in a case of subacute granulocytic leukemia (AG-2). See legend to Fig. 2.

and 8.21 μM per kilogram per hour. The mean value was 5.45, and 92 per cent of creatinine values were below normal. This case is an illustration of the occurrence of a low and variable creatinine excretion during the course of the disease. The creatine excretion, as shown in Fig. 4, varied between 0.12 and 2.37 μM per kilogram per hour. This amount of creatine is 2.1 to 35 per cent of creatinine excretion.

Discussion

The variability of creatinine and creatine excretion found in normal and leukemic children by Waisman and associates,[1] as against the consistency of creatinine excretion reported by Sandberg and co-workers,[2] suggests that a more detailed study is needed to elucidate the changes in and the role of these metabolites in leukemia. Although creatinine excretion values are frequently used to test the completeness of a urine collection, results reported in the literature suggest that this method should be applied only to an individual in whom the levels have been determined.[7] From values given by Garn and Clark,[8] the average creatinine excretion for men is calculated as 8.56 ± 1.32 μM per kilogram per hour. Van Pilsum and Wolin[9] reported that creatinine excretion is lower in individuals over 60 years of age. The average values for the creatinine excretion in children reported by Clark and associates[10] are quite constant for those children between the ages of 4 and 18 years. These authors show that the creatine excretion of the children is quite variable and, on the average, diminishes with age, and suggest that extremely large or nearly infinitesimal amounts would have to be excreted before it could be judged abnormal.

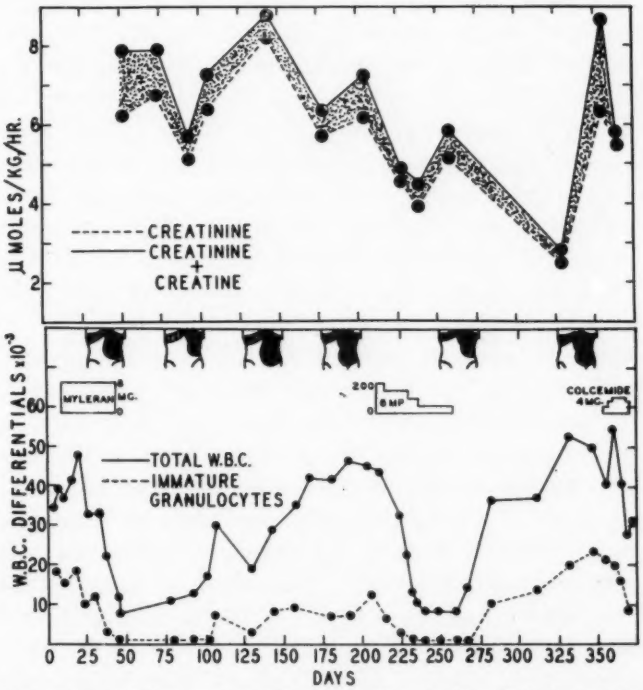


Fig. 4. Relation of creatinine and creatine excretion to the clinical and hematologic findings in a case of chronic granulocytic leukemia (CS-2). See legend to Fig. 2.

In the present study, excessive excretions of creatine were noted in several of the children with acute leukemia. The 54-year-old patient with subacute granulocytic leukemia also excreted very large amounts of creatine, and several with chronic leukemias excreted more creatine than would be expected of adults.

On the other hand, the excretion of creatinine was low in every case of leukemia that was followed. Although normal adults may excrete as little as 5.9 μM per kilogram per hour,[8] one would not normally expect 100 per cent of 15 individuals to have low creatinine outputs, with all but 3 excreting at times less than the above low amount. Thus the results reported in this paper suggest that the excretion of creatine and creatinine can be abnormal in leukemic patients.

Several reports in the literature further suggest abnormal creatinine and creatine metabolism in leukemia. Mitsuyama,[11] investigating the blood cells of leukemic patients, found the creatine concentration nearly twice normal. Since his work was on whole blood, the cells responsible for the excess creatine are unknown. However, Dinning and Day[12] have shown that, in monkeys recovering from a folic acid deficiency, the peak of creatine excretion corresponded to the time of maximum leukocyte numbers. Heaney and Eliel,[13] in studying the metabolic effect of 6-mercaptopurine in acute leukemia of a 3.5-year-old girl, reported excretion of creatinine as between 5.18 and 7.97 μM per kilogram per hour, with the highest value after she had been on the drug. Their control excretion level was 8.12 μM per kilogram per hour. Gerbrandy and associates[14] reported creatinine excretion values in leukemic patients quite comparable to those reported by us, 4.2 to 7.4 μM per kilogram per hour. They point out that there is a gradual fall in the creatinine excretion following the x-radiation of these patients, but the weight loss is such that the values reported in the above unit are not significantly changed. They found similar values in patients with cancer. Furthermore, Krauss[15] reported the blood creatinine concentration of a patient with chronic granulocytic leukemia as twice normal. In experimental mouse leukemia, Dinning and Seager[16] found a 3.5 fold increase in creatine excretion without changes in the creatinine excretion or the muscle creatine concentration. Their results suggest that creatine may play a significant role in leukocyte formation.

Thus, the previously reported findings on creatinine and creatine excretion in human leukemia are few and somewhat conflicting. Supporting evidence, however, points to abnormalities in the metabolism of these substances in leukemia. The exact role of creatinine and creatine remains unknown. Pathologic creatinuria is usually associated with muscular diseases, and Haberland and co-workers[17] suggest that the muscular weakness of mammals following ionizing radiation may be the cause of the creatinuria observed. One may attempt to explain this abnormal pattern of excretion of these metabolites with the observed curtailment of activities of the patients during their stay in the hospital. However, the existing information, in literature as well as in our findings, do not support the above. Actually, our studies indicate that the pattern of excretion is more closely correlated with the hematologic and clinical stage of the disease.

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The nature of the hemagglutinating agent noted in the blood serum of patients with viral hepatitis

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Animals were immunized against rhesus erythrocytes agglutinated with serum of patients with infectious hepatitis, and against sheep erythrocytes agglutinated by infectious mononucleosis sera. The resulting antisera were found to contain antiglobulin antibodies as shown by their ability to agglutinate human group O Rh-positive red cells sensitized by anti-Rh incomplete antibody. The presence of antiglobulin antibody in the immune rabbit sera indicates that the agglutinating agents in infectious hepatitis and infectious mononucleosis are globulin in nature, and the agglutination is considered to be due to antibody action rather than to the presence of a hemagglutinating virus.

The agglutination of *Macacus rhesus* erythrocytes by the blood serum of patients with infectious hepatitis was observed by the authors[1-5] and confirmed by Rubin and associates.[6] The studies reported here were designed to investigate the nature of the agglutinating agent.

The following two possibilities were considered: The material causing the reaction might be a virus present in the blood of the infected individual, or an antibody which appears in response to some feature of the disease and which reacts with the rhesus cell in a manner comparable to the anti–sheep cell agglutinin seen in infectious mononucleosis. If the latter were the case, the agglutinin, being an antibody, would be expected to fall in the class of gamma globulin, while if the reaction is due to a hemagglutinating virus, it would not have the characteristics of human gamma globulin. Since the active agent in the case of either mechanism is adsorbed on the agglutinated cell, immunization

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of rabbits with the agglutinated, washed rhesus cells should result in the formation in the rabbit of antibodies against both the rhesus cell and the antigenic protein adsorbed to it, whether virus or globulin.

If the adsorbed agglutinin be human gamma globulin, the antiserum against it would correspond to the antiglobulin or Coombs serum generally used to detect the presence of "incomplete" antibodies such as those seen in anti-Rh sensitization. Such an antiglobulin antiserum can be demonstrated by first sensitizing Rh-positive human erythrocytes of group O with a potent anti-Rh_o typing serum (slide type) containing no saline agglutinins, washing them 3 times in isotonic saline, and exposing the washed sensitized cells to dilutions of the immunized rabbit serum. Serum drawn before the period of immunization is used as a control. The presence of antiglobulin antibody is shown by the fact that such an antibody will cause clumping of the sensitized cells, which would remain suspended in its absence. The appearance of antiglobulin antibody after immunization would suggest that agglutination of the rhesus cell is due to a gamma globulin factor (antibody) in the patient with hepatitis, while the absence of such an antiglobulin antibody after immunization would favor the concept of the virus mechanism.

In a parallel study, sheep cells agglutinated by serum from individuals with infectious mononucleosis were washed and injected intravenously into rabbits in a manner similar to that described above. The resulting antiserum was also tested against the sensitized and washed group O Rh_o-positive human erythrocytes to demonstrate any antiglobulin antibody which might be present. Infectious mononucleosis serum was chosen for comparison, since it is a disease presumed to be viral in origin, which often shows hepatic involvement, and which is diagnosed by the Paul-Bunnell hemagglutination test using sheep cells.

Experimental procedure

Washed rhesus erythrocytes were agglutinated by sera from known cases of viral hepatitis previously shown to have agglutinating activity. The agglutinated cells were washed 3 times in isotonic saline and suspended to a concentration of approximately 5 per cent.

The rabbits chosen for immunization were bled by cardiac puncture and subsequently given 1.0 ml. of the agglutinated cell suspension via the marginal ear vein. A second injection of 1.0 ml. was given 1 week later. Eighteen days after the first injection the animals were bled and the serum specimens were tested for antiglobulin activity as described below.

Rabbits were bled by the cardiac puncture, the serum was removed, inactivated at 61° C. for 2 minutes, and tested for antiglobulin antibody in the following manner: human group O Rh_o-positive erythrocytes were sensitized by incubating for 30 minutes with an excess of human anti-Rh_o blocking serum. The cells were washed 3 times with isotonic saline and made up to a 2 per cent suspension. Serial dilutions of the sera were made in 0.2 ml. volumes and 0.2 ml. sensitized cells was added to each tube. After 30 minutes' incubation, the tubes were centrifuged at low speed and read after brisk shaking.

Results

The results are shown in Table I. These results demonstrate the presence of an antiglobulin antibody in the rabbits which is caused by the immunization with the agglutinated rhesus red cells and indicate that the agglutination is due to the

presence not of a virus but of a gamma globulin fraction which is assumed to be an antibody.

The same procedure was followed in a second group of animals, substituting for the rhesus cells sheep erythrocytes sensitized by the addition of serum from individuals with infectious mononucleosis. Results are shown in Table II.

Table I. Antiglobulin titers of rabbits immunized against hepatitis agglutinin

Rabbit antisera prepared against hepatitis agglutinin adsorbed on rhesus erythrocytes	*Rabbit antiserum agglutinating titer against human group O Rh_o erythrocytes*	
	Before immunization	*After immunization*
1. Hepatitis serum "3"	Negative 1:1	1:16
2. Hepatitis serum "27"	Negative 1:1	1:8
3. Hepatitis serum "Fuller"	Negative 1:1	1:32

Table II. Antiglobulin titers of rabbits immunized against infectious mononucleosis agglutinin

Rabbit antisera prepared against infectious mononucleosis agglutinin adsorbed on sheep erythrocytes	*Rabbit antiserum agglutinating titer against human group O Rh_o erythrocytes*	
	Before immunization	*After immunization*
1. Infectious mononucleosis serum "5"	1:2	1:128
2. Infectious mononucleosis serum "13"	Negative 1:1	1:32
3. Infectious mononucleosis serum "16"	1:4	1:64

The similarity of the responses elicited by the rhesus cells agglutinated by infectious hepatitis sera and by the sheep cells agglutinated by infectious mononucleosis sera indicates that the mechanism of agglutination is the same in each case and that this mechanism involves the activity of a gamma globulin fraction of the blood serum of the patient. In the case of the infectious mononucleosis serum, the agglutinating agent is known to possess the attributes of antibody, and our observations suggest that a similar antibody, rather than a virus, is responsible for the agglutination of the rhesus cells by infectious hepatitis serum.

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Prevalence of *Nocardia asteroides* in sputa examined by a tuberculosis diagnostic laboratory

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Over a two and one-half year period, 175 strains of suspected Nocardia asteroides were isolated from sputum after treatment with 4 per cent NaOH and mechanical shaking. All strains were later identified as N. asteroides. From 122 individuals, single isolations were made. From 12, various numbers of isolations were made, up to 21 on one individual. Lung biopsies were negative on 2 patients. A diagnosis of nocardiosis could not be made on any of the 134 patients. It is concluded that the mere isolation of N. asteroides from sputum is not a sufficient basis for establishing a diagnosis of nocardiosis.

In 1951, all routine cultural work for *Mycobacterium tuberculosis* by the Alabama State Health Department was terminated in the branch laboratories and transferred to the central laboratory. This centralization resulted in an increased demand for culturing so that the number of specimens increased rapidly to approximately 31,000 for the current year. Because of the belief that sodium hydroxide treatment was uniformly inhibitory to the growth of *Nocardia asteroides,* no attempt before 1957 was made to determine if this species could be isolated.

At that time, it was noted that Ajello and associates[1] had shown that *N. asteroides* generally survived digestion and concentration with sodium hydroxide and mechanical shaking. Accordingly, laboratory personnel were briefed on the colonial appearance of *N. asteroides* and instructed to pick and send all suspected isolates in pure culture for verification to the Mycology Unit

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of the Communicable Disease Center. The results of this study are presented here. All strains discussed in this paper were identified as *N. asteroides* except where noted.

Materials and methods

Sputa for culturing of *M. tuberculosis* were mailed from various areas of the state, including tuberculosis hospitals, in sterile, 1 ounce screw-capped containers. On arrival the specimens were treated with 4 per cent sodium hydroxide and shaken mechanically for 10 minutes. The sediment was then neutralized with 2 N HCl and centrifuged. Since spinal fluid specimens were mailed from long distances, they were treated with NaOH for 2 minutes and then neutralized and centrifuged. By means of capillary pipettes, representative samples of each specimen were smeared on 2 Löwenstein-Jensen slants and 2 tubes of blood agar[2] that contained 100 units of penicillin per milliliter. Smears were then made from the remainder of the concentrate. These were stained by Kinyoun's method, using 3 per cent alcoholic hydrochloric acid for decolorization. The cultures were incubated horizontally for several days at 35° C. and then placed in baskets for the remainder of the incubation period. They were checked every week after the second week, for a total of 8 weeks.

Results

When *N. asteroides* was present, its growth was usually apparent in 5 to 6 days. Under our routine the majority of cultures were not examined for 2 weeks, as noted above. The colonial coloration was quite variable. Pigmentation ranged from cream to pale yellow, orange, pink, coral, and brick red. Most of the isolates on original culture were a light tannish orange. They did not have the clear orange coloration of chromogenic unclassified mycobacteria. After 2 weeks of incubation the growth which appeared on most Löwenstein cultures was so scanty that it appeared only as a discoloration of the medium. Very few of the primary isolates were chalky white. Pigmentation varied in intensity and hue with subsequent transfers, age, and the media used for subcultures.

Nocardia-like colonies were picked and planted on Löwenstein's medium, blood agar, yeast-extract–glucose agar, and Sabouraud's dextrose agar. Smears were made from the colonies when growth appeared. They were examined after acid-fast staining. The mycelium of *N. asteroides* was very delicate and approximately the width of the cells of *M. tuberculosis*. It was acid-fast at least in part. The coccoid elements produced by this organism usually were acid-fast but the decision as to whether the isolate was acid-fast was based only on the staining properties of the mycelium. Colonies from Löwenstein's medium and blood agar were usually more acid-fast than those grown on yeast-extract–glucose or Sabouraud's dextrose agar. Regardless of acid fastness, isolates with delicate mycelium were isolated in pure culture and referred to the Communicable Disease Center Mycology Unit for identification. With this routine, approximately 94 per cent of the suspicious isolates were confirmed to be *N. asteroides* by methods described elsewhere.[3]

The majority of our 175 cultures were isolated on Löwenstein's medium (Jensen-Holm modification). However, 18 strains were isolated on the penicillin blood agar medium only, while 30 were recovered on both media.

All the nocardias were isolated from sputum except the few recovered from gastric washings. Two sputa were also positive for tubercle bacilli. *N. asteroides* was not detected in 78 sputa through examination of stained smears of both the unconcentrated and concentrated materials. However, 8 of these sputa yielded cultures of *N. asteroides*. Other investigators have reported finding *N. asteroides* on direct smear.[4]

During the two and one-half years of this study, 175 cultures suspected of being *N. asteroides* were isolated (Table I). Of the 168 cultures sent to the Communicable Disease Center, 151 were identified as *N. asteroides*. Three cultures were nonviable. Thus, approximately 94 per cent of the isolates were confirmed. Three of the cultures were designated as Nocardia species not *N. asteroides*. The identity of these 3 isolates will be reported elsewhere. Acid-fast chromogens were frequently concurrently present in the primary isolation tubes with *N. asteroides*.

Table I. Cultural results on clinical specimens examined for *M. tuberculosis* and *N. asteroides* (July, 1957, to August, 1960)

Analysis of clinical specimens	*No. of isolations*	*No. of cultures*
M. tuberculosis	10,197	
N. asteroides	175	
Single isolations	122	
Two isolations	7	
Three isolations	2	
Six isolations	2	
Twenty-one isolations	1	
Individual patients	134	
Total cultures forwarded to Communicable Disease Center		168
Confirmed *N. asteroides*		151
Negative for pathogenic fungi		11
Nonviable cultures		3
Nocardia species not *N. asteroides*		3
Repeat isolations not sent to Communicable Disease Center and identified by the Alabama State Department of Public Health	24	
Total No. of specimens cultured		85,435

Fifty-two of the patients from whom *N. asteroides* cultures were recovered were either suspected of having tuberculosis or were under treatment for that disease. In 37 patients, tuberculosis was ruled out. Data were insufficient or pending on the other patients.

The median age for those diagnosed as having tuberculosis was 58 years in white and 48 years in Negro men and 38 years for both white and Negro women. In those patients for whom a diagnosis of tuberculosis was excluded, the median age was 59 in white and 48 in Negro men. In the female patients the median ages were 58 and 52 in white and Negro, respectively.

The case histories of 4 elected patients are presented to illustrate the diagnostic problems presented by the isolation of *N. asteroides* from sputum.

Case reports

Case 1. A 59-year-old white farmer (F. W.) was admitted to the tuberculosis sanatorium on May 14, 1958, for diagnostic studies. He related a history of chronic cough and dyspnea. Chest x-ray disclosed displacement of the heart to the left with a fibrous reaction in the left lower lobe. Although numerous sputum studies were negative for tubercle bacilli, the tuberculin skin test was strongly positive. *N. asteroides* was isolated from the sputum in April, 1958. The patient was readmitted for special studies and a lung biopsy was performed on May 19, 1958. The pathologic findings were "nonspecific hemorrhage and fibrosis." A portion of the same tissue, examined by another pathologist, was reported as "chronic pneumonitis." *N. asteroides* has not been isolated from this patient since the original isolation from sputum. The patient continues to have chronic cough and dyspnea and is unable to work.

Case 2. Patient H. H. is of interest in that the final diagnosis of his illness was histoplasmosis. This 76-year-old white farmer was first admitted to the tuberculosis sanatorium on November 20, 1956. He gave a history of good health until 2 weeks before admission. At that time, after having cleaned a barn which had deposits of dried pigeon excreta, he developed dyspnea, a nonproductive cough, night sweats, fever, and mental confusion. Soon after this, he developed a sevère pleurisy in both lungs and was unable to work. His physician obtained a chest x-ray which revealed a "snow-storm" appearance in both lung fields. He was referred to the tuberculosis sanatorium since he was believed to have miliary tuberculosis. When first admitted to the sanatorium the patient was quite ill. The laboratory tests, which included a complete blood count and urinalysis, were noncontributory. The tuberculin skin test was positive (P.P.D. No. 2); the histoplasmin skin test was strongly positive. Complement-fixation studies revealed a histoplasma yeast-phase titer of 1:256 on January 18, 1957. Cultures for *Histoplasma capsulatum* were negative. The patient was discharged but was followed in the outpatient clinic and readmitted on several occasions for spinal studies. On June 25, 1958, a culture of *N. asteroides* was isolated from the sputum. He refused therapy for histoplasmosis because he continued to improve. The patient's chest x-ray had cleared up considerably by that time and continued to show additional clearing. All subsequent sputum smears and cultures have been negative for *N. asteroides* as well as for *H. capsulatum.* His chest x-ray now shows complete clearing. When last seen, he was in good health.

Case 3. A 62-year-old housewife (E. S.) was admitted to the tuberculosis sanatorium on January 14, 1959. She gave a history of having developed a severe "cold" in June, 1958, which was still present. The patient also had a harassing cough and dyspnea. She had consulted a physician, who made a diagnosis of "bronchitis." The patient was referred to the outpatient clinic of the sanatorium. Lesions were noted in the left upper lobe which appeared to represent minimal pulmonary tuberculosis with questionable activity. Three sputa were reported positive by smear for acid-fast bacilli. Because of this, the patient was admitted to the sanatorium for therapy. Subsequently, sputum cultures were reported positive for *N. asteroides* and *M. tuberculosis.* A tuberculin skin test with P.P.D. No. 2 and a histoplasmin skin test were positive on April 29, 1959. Complement-fixation titer with histoplasma yeast–phase antigen was positive at a dilution of 1:16 and the mycelium phase antigen was positive at a dilution of 1:8. This patient was treated for pulmonary tuberculosis. She was discharged on July 24, 1959, and has progressed well ever since. Sputum continues to be negative for both *N. asteroides* and *M. tuberculosis.*

Case 4. On this patient we now have 21 consecutive positive cultures of *N. asteroides,* collected over a 3 year period. He is a 56-year-old white man (C. H.) who was first admitted to the tuberculosis sanatorium on November 7, 1958. The patient stated that he was first told that he had pulmonary tuberculosis in 1951, on the basis of a routine chest x-ray. He was not treated at that time but was followed with frequent chest x-rays and sputum studies. His sputum was negative until 1955, at which time a positive sputum smear was reported by another laboratory. The patient refused treatment and continued to work as a farmer. In

the spring of 1958 he developed a chronic productive cough, dyspnea on exertion, tightness of the chest, and definite weakness. He tired easily and lost approximately 15 pounds of weight. Chest x-ray on admission to the sanatorium revealed contraction of the left upper lobe, with fibroexudative disease, and several areas of rarefaction, which were interpreted as cavities. The patient also had fibrous strands in the right upper lobe. Emphysema was noted in both lower lobes. He was classified as having far-advanced active pulmonary tuberculosis with pulmonary emphysema. Sputum smears on this patient were negative for acid-fast bacilli in our laboratory. However, *N. asteroides* was isolated from 8 out of 9 specimens submitted; no *M. tuberculosis* organisms ever were found. The patient left the sanatorium, contrary to medical advice, on October 23, 1958, but was readmitted on November 7, 1958. At that time it was noted that he had been placed on sulfadiazine in the outpatient

Table II. Source and chronological summary of *Nocardia asteroides* isolations on Patient C. H. over a 3 year period

Date	*Nocardia asteroides*	*Source*
1957.		
7/12	+	sputum
7/17	+	sputum
1958.		
6/17	+	sputum
6/19	+	sputum
6/26	+	sputum
6/27	+ (2)	sputum
7/1	+	sputum
7/7	+ (2)	sputum
7/31	–	sputum
8/29	+	sputum
11/13	+	sputum
11/25	+	sputum
12/31	+	sputum
1959.		
1/16	+	sputum
1/20	–	lung biopsy
1/20	–	mouth washings
5/1	+	sputum
5/4	+	sputum
5/5	+	sputum
1960.		
4/25	+	sputum
5/19	+	sputum
9/26	+	sputum

clinic. In addition to this, the patient had been on antituberculosis chemotherapy since June 23, 1958. *N. asteroides* organisms were isolated from 3 out of 4 specimens submitted at this time. The patient agreed to a lung biopsy and the operation was performed on January 21, 1959. The surgeon noted fine nodulations in the left upper lobe and obtained for microscopic study a tissue wedge which included these small granulation areas. The specimen secured was large enough that portions of it were submitted to the laboratory for culture, as well as histologic examination. The sanatorium pathologist reported a diagnosis of "chronic granuloma" of the lung. Hematoxylin and eosin, periodic acid–Schiff, and Brown and Breen stains were made of the tissue. No organisms were demonstrated. Cultures

were negative. This patient has been followed in the outpatient clinic and, with one exception, every specimen submitted for study to date has produced a luxuriant growth of *N. asteroides*. The clinical picture has shown little, if any, change to date. The chronologic sequence of isolations on this patient is shown in Table II.

Discussion

For purposes of classification, Runyon[5] proposed 4 distinct groups of previously unclassified acid-fast bacteria. The nocardias are included in Group IV. The organisms in this group characteristically are rapid growers. As previously noted, growth of *N. asteroides* becomes evident between 5 and 6 days. Its colony characteristics are distinctive and can be readily recognized with practice. This is borne out by the fact that approximately 94 per cent of the primary isolates of *N. asteroides* picked by visual inspection and staining were later confirmed by biochemical and cultural techniques.[3]

While all the *N. asteroides* isolates were at least partially acid-fast when stained by our routine method, other investigators have recommended less drastic destaining procedures. Among these methods are the use of weak sulfuric or aqueous hydrochloric acid destaining solutions. When using destaining methods less drastic when compared to our routine, we were not able to detect any significant difference. Nevertheless, we now routinely use both methods.

In 1951, Ajello and associates[1] determined the effect of various digestion and concentration procedures on *N. asteroides* and other fungi. While 84 per cent of the cultures were positive following treatment with 4 per cent sodium hydroxide plus mechanical shaking for 10 minutes, 100 per cent of the cultures were positive after 4 hour treatment with 10 per cent trisodium phosphate. Though direct inoculation of sputa without sodium hydroxide treatment could not be done concurrently in this series, the number isolated by digestion supports their observations. Several investigators[4, 6] have reported survival of *N. asteroides* after sodium hydroxide treatment. Our results are not compatible with reports by others[7-9] that sodium hydroxide treatment is invariably lethal to *N. asteroides* strains.

Attention should be called here to the use of trisodium phosphate as a digestant. Three sputa, in which *N. asteroides* was found after sodium hydroxide concentration, were negative when incubated overnight in trisodium phosphate. This lethal effect is probably similar to the toxicity of sodium hydroxide with long incubation periods observed by Ajello and associates[1] in their study. Because of its convenience, many laboratories use overnight incubation with trisodium phosphate rather than the 4 hour incubation period used by Ajello. The overnight incubation may account for failure to recover *N. asteroides*.

Obviously, we cannot infer from this study that all strains of *N. asteroides* which were present in the sputum specimens survived concentration. Nor do we suggest that sodium hydroxide treatment replace conventional direct culturing methods. While hospitals have access to fresh material, public health

laboratories do not. A much greater number of contaminants are present in our specimens and direct inoculation usually results in overgrowth by other organisms.

N. asteroides grows well on most common media. It is particularly important that this be kept in mind when culturing spinal fluid, since many routine media would demonstrate the organism, if present, without any special procedures.

The diagnosis of pulmonary nocardiosis on the basis of sputum findings is not easily established. In the literature, various opinions have been made as to the prevalence of the disease in this and other countries. Peabody and Seabury[4] have adequately discussed this problem. Some investigators have maintained that the mere recovery of *N. asteroides* from sputum is indicative of nocardiosis.[10, 11] On the basis of our findings, isolation of *N. asteroides,* without other supporting information, is an insufficient basis to establish a diagnosis for that disease. A diagnosis of pulmonary nocardiosis could not be established in any of the 134 patients from whom *N. asteroides* was isolated in the course of this study. Lung tissue from 2 patients was examined histologically and found to be free of *N. asteroides.* One of these patients (Case 4) has yielded 21 cultures of that organism over a 3 year period.

Since approximately 45 per cent of the specimens we routinely receive come from tuberculosis hospitals, we would, within the limits of our procedures, expect to find a much higher percentage of isolations of *N. asteroides* if McQuown's[9] observations were correct. McQuown estimated that approximately 5 per cent of patients in tuberculosis hospitals have nocardiosis. However, data were not presented to support this statement. As shown in Table I, in our study only 175 cultures of *N. asteroides* were obtained from 85,435 clinical specimens, or 0.2 per cent positive. The ratio of *N. asteroides* isolations to positive *M. tuberculosis* cultures was 1:58.

Of the 175 strains of *N. asteroides* isolated, 42 were tested for pathogenicity. All of them were found to be pathogenic to the guinea pig. With some strains it was necessary to inoculate several guinea pigs before pathogenicity could be demonstrated.

The relatively large number of recoveries of *N. asteroides* from sputum raises the question as to the significance of this fact. The majority of the patients with positive cultures had diagnoses of pulmonary disorders of undetermined etiology. Despite the failure to demonstrate histologically the presence of *N. asteroides* in lung tissue from 2 of the patients, it may be that this group of individuals was afflicted with a chronic, mild form of nocardiosis. Intensive bacteriologic studies to exclude infection by other microorganisms and histologic studies would need to be carried out to determine the truth of this.

If the patient's symptoms were induced by other agents or causes, the possibility exists that *N. asteroides* was a secondary invader. Such was probably the case in the patient with histoplasmosis and in those from whom *M. tuberculosis* had been isolated.

Two potentially pathogenic organisms, *Actinomyces bovis* and *Candida albicans,* are considered to be normal components of the microflora of the human

body. They are recovered from normal individuals with great frequency. It may be that *N. asteroides* normally exists in the body in a nonpathogenic state. However, this possibility is considered to be remote, since *N. asteroides* was recovered from only 175 of 85,435 sputa. It would seem that *N. asteroides* is not normally present in the human body.

N. asteroides has been recovered from soil,[12-15] and is considered to exist there as a saprophyte. Inhalation of the air-borne mycelial elements of this actinomycete is generally the source of primary and secondary infections. A cultural survey of sputum, and bronchial and gastric washings obtained from individuals free of pulmonary disease would need to be carried out to determine whether *N. asteroides* is normally present in the human body.

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Hexosamine content of aged and chronically diseased lung

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Connective tissue ground substance forms a substantial portion of lung tissue and may be implicated in the development of pulmonary emphysema and fibrosis. Hexosamine, an important constituent of ground substance, and its components, galactosamine and glucosamine, were determined in lung tissue from laboratory animals of varying ages and human necropsy material. Each specimen was graded histologically. Correlations were made with the clinical course and autopsy material.

The mean pulmonary hexosamine content in healthy animals of several species extended from 0.771 to 0.583 mg. per 100 mg. of tissue dry weight and averaged 0.652 mg. for normal human lung. The mean ratio of galactosamine to glucosamine ranged from 0.19 at age 2 months to 0.16 for 2-year-old rabbits, and averaged 0.223 in man. Variation within a species proved to be small, and the absence of significant change throughout a broad life span of laboratory animals suggested that age-dependent hexosamine alterations were unlikely in lung tissue.

Fifteen specimens, characterized histologically as pulmonary emphysema, averaged 0.671 mg. per cent for hexosamine content and 0.249 for the galactosamine-glucosamine ratio, compared to means of 0.782 and 0.170, respectively, for 18 specimens exhibiting the microscopic findings of acute infection, hemorrhage, or edema.

These studies demonstrated that galactosamine-glucosamine ratios were considerably higher than control values in certain emphysema specimens, although group differences were not significant statistically. In the presence of acute histologic change, however, the mean hexosamine content increased, and the average galactosamine-glucosamine ratio decreased. These differences were statistically significant. It is suggested that these hexosamine changes may have been associated with basic alterations in lung ground substance that could contribute to the development of chronic pulmonary disease. An alternative explanation is that the bioochemical findings reflected differences in pulmonary blood content.

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Pulmonary emphysema is a common disabling illness, but the pathogenesis is not completely understood. The prevalence of this disease either in the aged or in younger patients with preexistent chronic bronchopulmonary disorders suggests that allied degenerative processes may initiate pulmonary emphysema. Recent investigations of these relationships have included analysis of lung tissue for evidence of age- or disease-dependent biochemical changes. Pierce and Hocott[1] have reported decreased collagen-elastin ratios in aged lung but have not observed significant differences in collagen content between aged and emphysematous lung. Briscoe and associates[2] have observed a progressive increase in lung hydroxyproline content which correlates well with age, implying a related increase in collagen as well. Similar changes for lung collagen have been reported in animals.[3]

Hexosamine has been demonstrated to change significantly in aged cartilage and nucleus pulposus, but similar observations have not been made on lung tissue.[4, 5] For that reason, pulmonary parenchyma was studied to determine whether hexosamine or its primary components, galactosamine and glucosamine, were altered in the presence of aging or disease.

Methods

Lung parenchyma was obtained from healthy rats, dogs, guinea pigs, and rabbits of varying ages. Human pulmonary parenchymal tissue specimens were obtained at necropsy. Histologic and biochemical observations were made on each specimen.

The portion for histologic study was fixed in buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin, the Verhoeff-Van Gieson elastic tissue stain, and the Alcian blue periodic acid–Schiff mucopolysaccharide strain. The specimens from human material were classified microscopically into the following 4 categories: (1) normal; (2) emphysema and fibrosis; (3) acute changes of edema, congestion, pneumonia, hemorrhage, or combinations thereof; and (4) mixed acute, chronic inflammation, and emphysematous changes.

The tissue for biochemical analysis was acteone dried, weighed, hydrolyzed in dilute acid, filtered, and placed on short Dowex–50 ion exchange resin columns. Hexosamine was eluted off with strong acid and measured colorimentrically.[6] Lung specimens from perihilar areas contained significantly larger amounts of hexosamine than did peripheral aliquots. The differences seemed to reflect the greater bronchial cartilage content of proximal tissues. For this reason specimens were obtained routinely from peripheral subpleural segments of the lower lobes. A portion of the same hydrolysate was placed on long Dowex–50 resin columns, and the glucosamine-galactosamine components were separated by a time acid gradient elution into serial 2 ml. fractions.[7] The first component appeared after 120 ml. acid had been eluted, the second after 160 ml. had been separated. Mixing of components at their junction during the fraction collection was readily estimated by mechanical planimetry of the plotted curves, and good separation was usually obtained. The sum of collected fractions ranged from 90 to 115 per cent of the total hexosamine value determined directly from the same specimen, and the maximum observed variation in galactosamine-glucosamine ratios was less than 10 per cent when a fractionation was repeated.

Normal tissues in animals and man were analyzed for hexosamine, galactosamine, and glucosamine differences related to age or species. The biochemical observations on pathologic human lung were correlated with specimen histology and available clinical data.

Results

The average hexosamine content of healthy adult mammalian lung tissue was 0.771 mg. per 100 mg. dry weight for rats, 0.704 for dogs, 0.583 for rabbits, 0.642 for guinea pigs, and 0.652 for man (Table I). Galactosamine-glucosamine ratios were 0.16 and 0.223 for rabbits and humans, respectively.

Table I. Lung hexosamine, galactosamine, and glucosamine observations from healthy adults of several mammalian species

Species	*Number of observations*	*Average hexosamine content (mg. per 100 mg. dry weight)*	*Galactosamine to glucosamine ratio*
Rats	9	0.771	
Dogs	6	0.704	
Rabbits	6	0.583	0.16
Guinea pigs	11	0.642	
Man	9	0.652	0.223

Table II. Age distribution of lung hexosamine and its components in a healthy homogenous strain of laboratory rabbits

Age of rabbits	*Number of observations*	*Average hexosamine (mg. per 100 mg. of tissue dry weight)*	*Galactosamine to glucosamine ratio*
Newborn	3	0.503	
2 weeks	3	0.521	
2 months	3	0.557	0.19
4 months	6	0.550	0.18
8 months	3	0.541	0.23
18 months	3	0.566	0.19
2 years or older	6	0.583	0.16

In order to evaluate the effect of aging, lung tissue hexosamine was determined in a pure strain of healthy laboratory rabbits varying in age from birth to more than 2 years (Table II). The hexosamine content ranged from an average of 0.503 mg. per cent at birth to 0.583 for animals greater than 2 years of age. Average galactosamine-glucosamine ratios for the same rabbit specimens varied from 0.16 to 0.23.

Pulmonary parenchymal specimens obtained at necropsy from 51 patients with pulmonary abnormalities were studied. Hexosamine findings were tabulated according to both histologic and clinical criteria. Specimens from 15 patients revealed the microscopic changes of emphysema only. In many instances there was clinical documentation of respiratory disability, including dyspnea, impaired pulmonary function, anoxemia, and hypercapnia. The histologic abnormalities consisted of alveolar space dilatation, alveolar wall narrowing, elastic fiber fragmentation, avascularity, and rupture or fibrosis of septal walls (Table III). Histochemically, the amount of elastic tissue fibers visible seemed unchanged from the normal, although the character and continuity

did change remarkably. The quantitative and qualitative distribution of acid mucopolysaccharide, as visualized with the Alcian blue P.A.S. strain, did not differ from the findings in normal lung tissue. The mean hexosamine content of emphysematous lung was 0.671 mg. per 100 mg. dry weight with an average galactosamine-glucosamine ratio of 0.249, and it did not differ significantly from normal values. However, 8 specimens showed a rise in the galactosamine-glucosamine ratio of more than one standard deviation above normal values, up to levels as high as 0.34.

Specimens from 18 subjects showed acute histologic changes only, including edema, hemorrhage, or infection (Table IV). In this group, the mean hexosamine content of 0.782 mg. per cent did differ significantly from that observed in the normal or emphysema groups. The average galactosamine-glucosamine ratio of 0.170 was more than one standard deviation below the normal mean in 11 of the 18 specimens.

Eighteen specimens demonstrated, histologically, a mixture of acute, chronic inflammatory, and emphysematous changes. Hexosamine and its components did not differ quantitatively from the normal range in this group.

Table III. Lung specimens obtained from 15 patients with histologic findings of pulmonary emphysema and fibrosis

	Pure pulmonary emphysema (mg. per 100 mg. tissue dry weight)			
Histologic grade	*Hexosamine*	*Galactosamine*	*Glucosamine*	*Galactosamine to glucosamine ratio*
1+	0.573	0.149	0.424	0.26
1+	0.533	0.171	0.362	0.32
1+	0.592	0.154	0.438	0.26
1+	0.709	0.184	0.525	0.26
1+	0.608	0.176	0.432	0.29
2+	0.607	0.174	0.433	0.26
2+	0.675	0.169	0.506	0.25
2+	0.817	0.139	0.678	0.17
2+	0.743	0.119	0.624	0.16
2+	0.730	0.161	0.569	0.22
2+	0.802	0.201	0.601	0.25
3+	0.716	0.122	0.594	0.17
3+	0.640	0.147	0.493	0.23
4+	0.610	0.207	0.403	0.34
4+	0.710	0.206	0.504	0.29
Mean	0.671	0.165	0.506	0.249
Standard deviation	0.084	0.026	0.089	0.055

Discussion

It is apparent that normal lung tissue contains relatively small amounts of hexosamine, approximating 0.6 per cent of tissue dry weight. Although small species differences occur, the magnitude of this variability is less than 20 per cent of the total when small mammals are compared to man.

There is also little variation in the quanitative distribution of hexosamine components in the normal animal and human lungs studied. Furthermore, these components, expressed as the ratio of galactosamine to glucosamine, did not differ quantitatively throughout a broad life span of healthy rabbits. It seems likely that this observation can be extended to healthy human lung as well, and that changes in hexosamine content or components do not appear in normal or aging lung in the absence of histologically evident disease. Changes in this measurement are reported for other tissues in the presence of the microscopic aging changes. For example, in aging nucleus pulposus, the hexosamine content and the galactosamine-glucosamine ratio decrease sharply.[4]

Table IV. Lung specimens obtained from 18 patients with the histologic findings of acute infection, hemorrhage, edema, or a combination thereof

	Acute histologic changes only (mg. per 100 mg. tissue dry weight)			
Histologic grade	*Hexosamine*	*Galactosamine*	*Glucosamine*	*Galactosamine to glucosamine ratio*
2+	0.911	0.109	0.802	0.12
3+	0.770	0.177	0.593	0.23
3+	0.829	0.058	0.771	0.07
4+	0.528	0.069	0.459	0.13
2+	0.832	0.125	0.707	0.15
4+	0.935	0.318	0.617	0.34
4+	0.912	0.192	0.720	0.21
3+	0.597	0.131	0.466	0.22
2+	0.667	0.093	0.574	0.14
3+	0.952	0.133	0.819	0.14
2+	0.556	0.178	0.378	0.32
2+	0.596	0.066	0.530	0.11
1+	0.675	0.142	0.533	0.21
4+	0.934	0.205	0.729	0.22
2+	0.913	0.055	0.858	0.06
1+	0.853	0.145	0.708	0.17
2+	0.793	0.111	0.682	0.14
4+	0.824	0.066	0.758	0.08
Mean	0.782	0.132	0.650	0.170
Standard deviation	0.14	0.063	0.014	0.077

The trend toward higher ratios of galactosamine to glucosamine in emphysematous lung suggested the possibility of a significant relationship between altered hexosamine components and the development of pulmonary emphysema. In this regard, Fessler[8] has demonstrated striking elastic properties for hyaluronic acid-collagen-water mixtures. He has also reported that variation in the small amount of hyaluronic acid present will alter these same elastic properties substantially. Since changes in lung elasticity seem important in the pathogenesis of pulmonary emphysema, it is possible that even small hexosamine changes, as were observed in emphysematous lung, could modify the mechanical properties of pulmonary tissue and lead to subsequent morphologic abnormalities.

It is also possible that the same changes in lung galactosamine and glucosamine components may reflect variations in pulmonary blood content at the time tissue is obtained for analysis. Blood contains relatively large amounts of hexosamine with a very low galactosamine-glucosamine ratio, approximating 100 mg. per cent and 0.10, respectively.[9] Therefore, a decrease in lung blood content might result in an apparent rise in the galactosamine portion of total lung hexosamine. Since the more severe grades of emphysema are accompanied by microscopic avascularity, this possible explanation merits serious consideration.

Significant changes in lung hexosamine and its components were observed in the presence of acute histologic findings. These alterations represented the opposite of trends noted for emphysematous tissue. Since all acute histologic changes included the appearance of large amounts of blood or its products in lung parenchyma, plasma hexosamine could be responsible for both the high tissue hexosamine and the low galactosamine-glucosamine ratios actually observed. However, Gersh[10] has observed electron microscopic changes in lung basement membrane during acute inflammation. Our observations may be related to similar ground substance changes.

Although the causes of emphysema remain obscure, it appears probable that primary connective tissue structural changes produce the physiologic alterations of this illness. Lung is unique among the body organs which change size repetitively as an integral part of their function, in that slender strands of connective tissue, cartilage, negative intrapleural pressure, and surface forces fulfill the structural requirements usually accomplished by large groups of muscle or bone. It is, therefore, of considerable importance to delineate alterations in supporting tissues which might produce irreversible morphologic and functional changes. The lung hexosamine abnormalities observed in this study may be associated with the histologically evident disease, but could also have been an incidental finding due to changes in pulmonary blood content. Further investigation of isolated lung structures, such as bronchial cartilage, and an evaluation of other pulmonary connective tissue components, may clarify this problem.

Acknowledgment is made of the technical assistance of Mary Rives and Mrs. Josephine Smith.

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Announcement

Beginning with Volume 58, THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE will no longer publish both an abstract at the beginning and a summary at the end of each article. We will publish a single abstract of 250 words or less describing in general terms the problem under investigation, the methods employed, the observations which have been made, and the principal conclusions.

Laboratory methods

A method for the localization of catalase on starch gels

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WITH THE TECHNICAL ASSISTANCE OF FLORINE FITZWATER and HELEN HUGHES

The rapid rate of destruction of hydrogen peroxide by the enzyme catalase was exploited in the development of a method by which the enzyme can be localized on a starch gel after electrophoresis. Following a 30 second immersion in a solution of sodium thiosulfate and hydrogen peroxide, the gel is removed to a solution of potassium iodide, and the staining reaction occurs in the next few minutes. Reaction between sodium thiosulfate and hydrogen peroxide is prevented by destruction of hydrogen peroxide in the area of the gel containing catalase. On the surface of the rest of the gel, hydrogen peroxide destroys the reducing capacity of sodium thiosulfate and oxidizes the iodide to iodine. The starch gel is stained the characteristic blue-black color by iodine in all areas except that containing catalase.

Data are presented regarding the sensitivity of the method.

Catalase is perhaps best known for the remarkable speed with which it decomposes hydrogen peroxide to molecular oxygen and water. One molecule of catalase can destroy 44,000 molecules of hydrogen peroxide per second. It ranks as one of the most effective enzymes known.[1]

This characteristic of catalase was exploited in the present investigation to develop a technique by which the enzyme can be localized on a starch gel after electrophoresis of either hemolysates of human erythrocytes or commercially available bovine catalase. The purpose of this report is to present the details of the development of the staining method used in earlier studies reported by us.[2]

Methods

Electrophoresis. The method of starch gel electrophoresis described by Smithies[3] was used. Electrophoresis was carried out for 5 to 8 hours at room temperature with a potential gradient of 4 v. per centimeter and a current of 4 to 5 Ma. for each gel when gels

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2 by 25 by 0.7 cm. in size were used and for 10 to 20 hours at room temperature with a potential gradient of 4 to 5 v. per centimeter and a current of 20 Ma. when a gel 15.5 by 26 by 0.7 cm. in size was used.

Hydrolyzed starch was obtained commercially.* Borate buffer was employed at pH 8.6 and prepared as follows:

BRIDGE BUFFER. (Approximately 0.3 M) 18.55 Gm. H_3BO_3 was dissolved in distilled water. The desired pH was obtained by the addition of concentrated NaOH solution and the volume brought to 1 L. by the addition of distilled water.

GEL BUFFER. (Approximately 0.03 M) 1.42 Gm. H_3BO_3 was dissolved in distilled water. The desired pH was obtained by the addition of concentrated NaOH solution and the volume brought to 1 L. by the addition of distilled water. As the starch is slightly acid, the pH of the gel buffer must be slightly higher than that of the bridge buffer. The gel must have the same final pH as the bridge buffer.

Staining materials

Catalase stain.

SOLUTIONS.

H_2O_2	0.88 M	(3.0 per cent)
$Na_2S_2O_3 \cdot 5H_2O$	0.06 M	(1.5 per cent)
KI	0.09 M	(1.5 per cent)

The gel was cut along its horizontal plane with a razor and the 2 pieces were separated. The first staining solution was prepared by pouring 12 ml. of $Na_2S_2O_3 \cdot 5H_2O$ (0.06 M) and 28 ml. of H_2O_2 (0.88 M) together into a small flat dish. The piece of gel to be stained was immediately immersed in this solution for 15 to 30 seconds. With a spatula the gel was removed from the first solution and placed into a second dish, which contained 50 ml. of KI (0.09 M). The dish was gently rocked and the blue-black staining of the starch by iodine appeared after a few moments at the anodal end of the gel. The staining of the starch proceeded slowly until all but the area of catalase activity was stained. This area was well defined before maximum intensity of the stain was obtained. When suitable differentiation was obtained, the gel was removed and washed in water redistilled in glass. The gels were preserved for short periods without distortion when placed on a sheet of glass or plastic, covered with a thin sheet of polyethylene, and stored in a Deepfreeze. Stored in this manner, they gradually dried out, and there was some distortion. Photographs were taken immediately after staining or within 12 to 15 hours of storage.

Benzidine-peroxidase stain. Benzidine base (0.5 Gm.) was ground with a few drops of glacial acetic acid. Two hundred milliliters of water was added and the solution filtered. To the filtrate was added 4 drops of 0.88 M hydrogen peroxide. Freshly prepared solutions were used and the gels were immersed for 5 minutes.

It is important to use water redistilled in glass in making all buffers and staining solutions because ordinary distilled water may contain reducing substances which interfere with the catalase staining reaction.

Results

When a starch gel was immersed in a solution of hydrogen peroxide after electrophoresis of red blood cell hemolysate, a gas escaped from the surface of the gel in the area behind the main hemoglobin band. Within 1 to 2 minutes, as the peroxide solution permeated the gel, some gas bubbles which formed were trapped within the substance of the gel (Fig. 1, *B*). After exposure to the hydrogen peroxide solution for 2 to 3 minutes, the gel was removed to a 0.1 N potassium permanganate solution. The area of the gel

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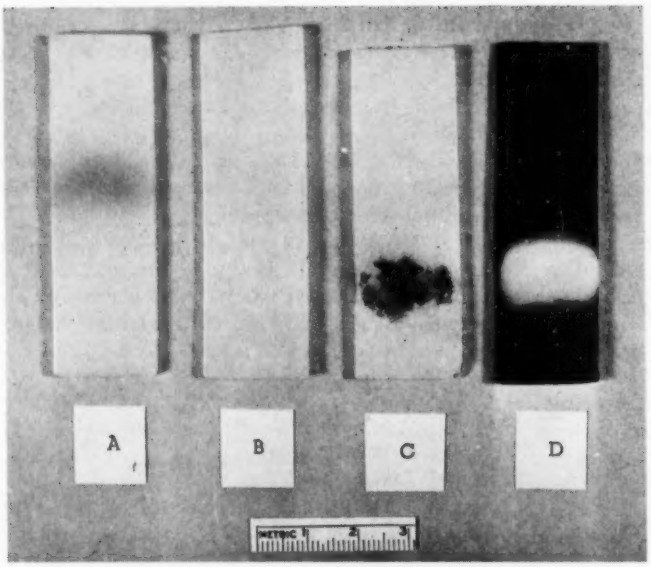

Fig. 1. Electrophoresis of erythrocyte hemolysate with hemoglobin concentration 5.0 Gm. per cent. Each gel was divided lengthwise and the halves treated as follows: *A*, unstained starch gel; *B*, after immersion in H_2O_2 (3 per cent) for 3 minutes. Note fine bubbles of trapped gas; *C*, immersion in H_2O_2 (3 per cent) followed by 0.1 N $KMnO_4$ and rinsed with one per cent acetic acid; and *D*, stained with catalase stain described in this paper.

containing the trapped gas was stained the purple-red color of oxidized potassium permanganate, while the rest of the surface of the gel was covered with a brown precipitate of manganese dioxide. This contrast was made more prominent by rinsing the gel in a solution of 0.16 M acetic acid, which removed the manganese dioxide and revealed that the potassium permanganate, in its oxidized state, had permeated the depth of the gel in the catalase area. The rest of the gel was colorless (Fig. 1, *C*). The fact that molecular oxygen is released when catalase acts upon hydrogen peroxide suggested that the bubbles of gas were due to the presence of catalase which had been electrophoretically separated from hemoglobin.

The marked distortion of the leading edge of the catalase band which resulted when this stain was used made it desirable to develop a stain which would more accurately define the area of catalase activity. The stain described above under *Methods* depends upon the ability of catalase to destroy hydrogen peroxide and upon the reaction between hydrogen peroxide and sodium thiosulfate. It clearly defines the area of catalase activity (Fig. 1, *D*).

The reaction between sodium thiosulfate and hydrogen peroxide is a complicated one. The products of the reaction probably include sulfur dioxide, sulfur, and sodium tetrathionate. The important fact for our purpose is that this reaction destroys the effectiveness of sodium thiosulfate as a reducing compound. When equal parts of 0.88 M hydrogen peroxide solution and 0.06 M sodium thiosulfate solution are mixed, enough sodium thiosulfate

remains to reduce added iodine to iodide for only one minute. Thereafter, it will fail to reduce added iodine. It is the relatively rapid destruction of sodium thiosulfate as a reducing compound that makes it important to mix the sodium thiosulfate solution with the hydrogen peroxide *immediately* before using.

Electrophoresis of commercially available bovine liver catalase and horse-radish peroxidase was carried out using starch gel as the supporting medium with a borate buffer at pH 8.6. The components of the horse-radish peroxidase were identified by the benzidine stain, but not by the catalase stain. Conversely, the bovine liver catalase was easily identified with the catalase stain, but it could not be located by the use of the benzidine stain (Fig. 2).

Electrophoresis of a hemolysate obtained from a person with normal adult hemoglobin was carried out side by side with a solution containing bovine liver catalase using a single starch gel as a supporting medium and borate buffer at pH 8.6. The location of each catalase was determined using the method described in this paper (Fig. 3). Under these conditions the mobilities of the 2 catalases were found to be different and erythrocyte catalase was found to trail the major hemoglobin component.

To determine the sensitivity of the method, serial dilution of bovine catalase and fresh human hemolysates were used. This procedure disclosed

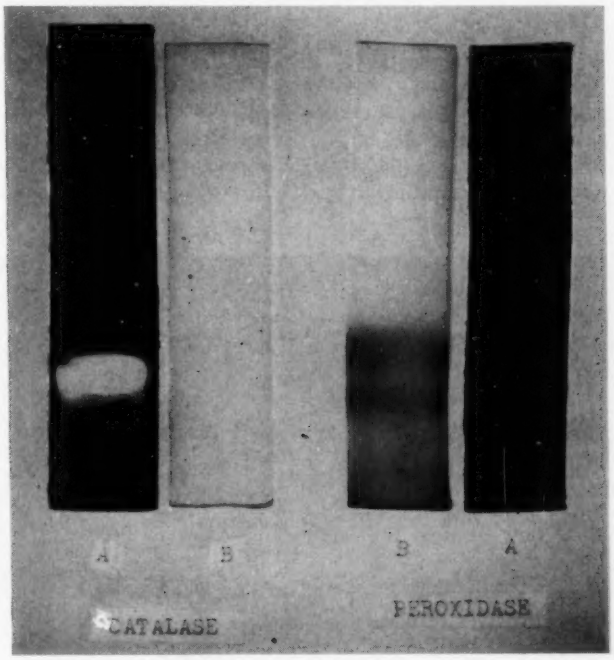

Fig. 2. Comparison of the staining characteristics of bovine liver catalase and horse-radish peroxidase. *Catalase.* The 2 halves of the gel containing catalase were stained as follows: *A,* with catalase stain described in this paper; *B,* with the benzidine stain. *Peroxidase.* The 2 halves of the gel containing peroxidase were stained as follows: *B,* with the benzidine stain; *A,* with catalase stain described in this paper.

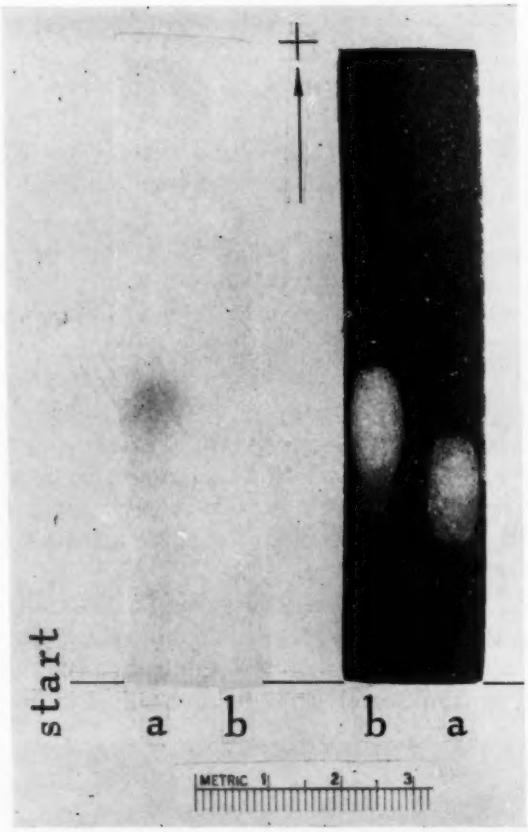

Fig. 3. a, Approximately 0.01 ml. of normal adult erythrocyte hemolysate (Hb—8.4 Gm. per cent) and *b,* approximately 0.01 ml. of bovine liver catalase (5 mg. per milliliter of phosphate buffer, pH 7.0) were run side by side on the same gel, pH 8.6, for 5 hours. Gel was cut and one half left unstained (left) while the other (right) was stained for catalase activity.

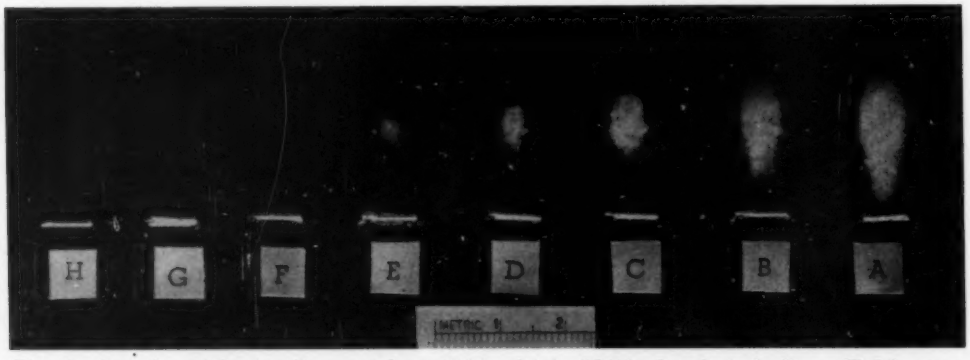

Fig. 4. Samples of bovine liver catalase (0.01 ml.) in M/15 phosphate buffer, pH 7.0, were applied to filter papers and inserted as indicated. Doubling dilutions were used. The solution applied to filter paper inserted at *A* contained 16.6 μg bovine liver catalase while that applied to the filter paper inserted at *E* contained 1.0 μg. Catalase stain described in the text was employed to detect site of catalase activity after vertical electrophoresis for 10 hours with potential drop of 4 to 5 v. per centimeter and current of 20 Ma. using borate gel buffer, pH 8.6.

that a quantity as small as 1.0 μg of bovine liver catalase (Fig. 4) or the amount of erythrocyte catalase in 0.01 ml. of fresh human erythrocyte hemolysate with a hemoglobin concentration of 0.078 Gm. per cent and K catalase 5.45[4] could be detected by the method (Fig. 5).

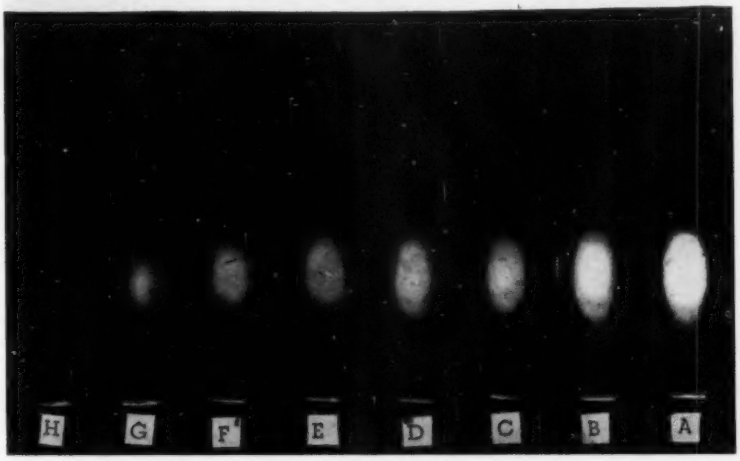

Fig. 5. Samples (0.01 ml.) of red cell hemolysate from an individual with K catalase value of 5.45 units determined by the assay method of Takahara and associates[4] were applied to filter papers and inserted as indicated. Doubling dilutions were used. The hemolysate applied to filter paper inserted at *A* contained 1.0 mg. hemoglobin while that applied to the filter paper inserted at *H* contained 0.0076 mg. hemoglobin. Catalase stain described in text was employed to detect site of catalase activity after vertical electrophoresis for 20 hours with potential gradient of 4 to 5 v. per centimeter and a current of 20 Ma. using a borate gel buffer, pH 8.6.

Discussion

It seems reasonable to assume that the solution of sodium thiosulfate and hydrogen peroxide penetrates the surface of the gel slightly during its short immersion. In the area occupied by the catalase, hydrogen peroxide is rapidly destroyed while the sodium thiosulfate is left unaltered. On the rest of the surface of the gel sodium thiosulfate is destroyed by the excess hydrogen peroxide. When the gel is immersed in the potassium iodide solution, the hydrogen peroxide present on the surface of the gel, except in the area of catalase activity, oxidizes the iodide to iodine and the starch is stained a characteristic blue-black color. As the hydrogen peroxide is destroyed in the catalase area, no iodine is released there, and any iodine which might escape into the solution from the rest of the gel and settle there is reduced on contact by the sodium thiosulfate present.

This technique may be used to localize catalase in any medium containing starch or to which starch might be added.

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A new automatic carbon dioxide analyzer: Its performance in presence of anesthetic gases and vapors

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A method for the rapid automatic measurement of carbon dioxide concentration of respiratory gases is described. The determination is based upon the changes in pressure of a sample of known volume (45 ml.) after removal of carbon dioxide by adsorption on soda lime. Progression of gas flow through the apparatus is effected by a rocking, nearly frictionless mercury pump, and requires 15 seconds for completion. Carbon dioxide in air or oxygen, in concentrations of 2 to 14 per cent, was measured with an accuracy of ± 0.1 vol. per cent. In the presence of anesthetic gases or vapors, an accuracy of ±0.2 vol. per cent was obtained. Wet or dry samples were measured with equal accuracy, and rapid changes in room temperature, within the range of 15 to 25° C., had no effect on accuracy.

The clinical diagnosis of disturbances in ventilation continues to be handicapped by the lack of a simple and accurate method for the analysis of carbon dioxide concentration of respiratory gases. This need can be met largely by a new automatic carbon dioxide analyzer,† designed on old principles, but incorporating special advantages, particularly for use in the presence of anesthetic gases and vapors. A report of its performance is presented.

Principle of operation

The gas sample is automatically drawn by a rocking spring-loaded mercury pump through a cartridge of saturated crystals of ammonium sulfate into a constant volume chamber, adjusted to atmospheric pressure, and forced through a cartridge of soda lime. The ammonium sulfate brings the moisture content of the sample to approximately 85 per cent and in equilibrium with the moisture of soda lime; the soda lime then removes the

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†Developed by Dewey and Almy Chemical Division, W. R. Grace & Co., Cambridge, Mass.

carbon dioxide, causing a drop in pressure proportional to the carbon dioxide content of the sample. This drop in pressure is measured by a sensitive pressure gauge which is calibrated to read as carbon dioxide in volumes per cent of the original sample. In order to avoid contamination of a sample by gas from the previous analysis remaining in the ammonium sulfate cartridge and the associated tubing, the instrument automatically goes through its operating cycle twice. A sample of approximately 45 ml. is required and the entire analysis takes 15 seconds.

Fig. 1. Automatic analyzer showing cartridges of soda lime and ammonium sulfate, pressure gauge, and handle for initiating automatic analysis. Manual depression of handle winds up spring which drives rocking pump.

Methods

Performance was tested with concentrations of carbon dioxide of 2 to 14 per cent both with and without anesthetic gases and vapors. The analyzer response to anesthetic gases or vapors alone or with oxygen was also tested. The mixtures of carbon dioxide and oxygen used were checked repeatedly by the standard method of Haldane.[1] The mixtures of carbon dioxide, anesthetic gases or vapors, and oxygen (or air) were made in a 5 L. Cryovac plastic bag* using a 50 or 100 ml. syringe and a threeway stopcock. It was not possible to check the accuracy of such mixtures by Haldane analysis, since anesthetic gases are rapidly adsorbed by alkali and result in a large positive error. However, to test the method of syringe mixing, mixtures of carbon dioxide and oxygen or air were made, analyzed on the Haldane apparatus, and found to agree with predicted carbon dioxide concentrations

*All analyses were made within 10 minutes. At the end of 30 minutes, carbon dioxide concentrations within such bags were found to fall approximately 0.1 vol. per cent.

within 0.25 vol. per cent. To test the effect of humidity, wet and dry mixtures of carbon dioxide and oxygen were tested. Performance with moderate and marked changes in temperature was also examined.

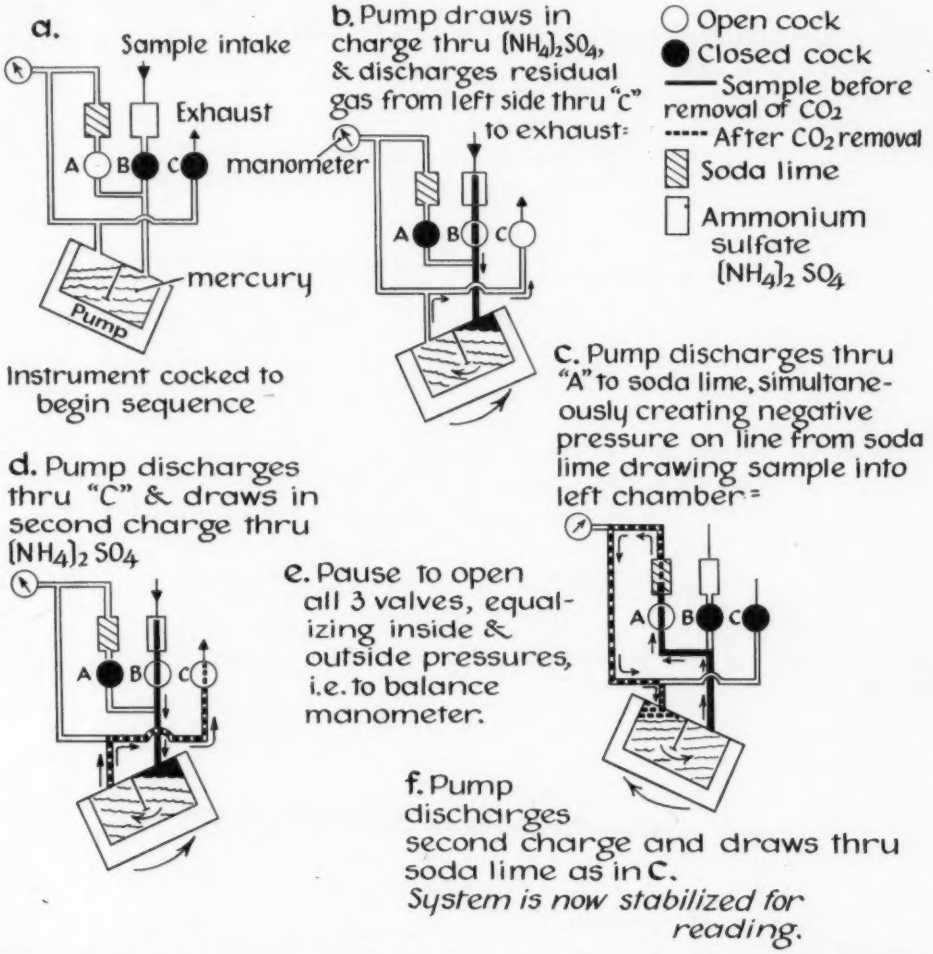
a.
Sample intake
Exhaust
A B C
manometer
mercury
Pump
Instrument cocked to begin sequence
b. Pump draws in charge thru $(NH_4)_2SO_4$, & discharges residual gas from left side thru "C" to exhaust
A B C
Open cock
Closed cock
Sample before removal of CO_2
After CO_2 removal
Soda lime
Ammonium sulfate $(NH_4)_2SO_4$
c. Pump discharges thru "A" to soda lime, simultaneously creating negative pressure on line from soda lime drawing sample into left chamber
d. Pump discharges thru "C" & draws in second charge thru $(NH_4)_2SO_4$
A B C
e. Pause to open all 3 valves, equalizing inside & outside pressures, i.e. to balance manometer.
A B C
f. Pump discharges second charge and draws thru soda lime as in C.
System is now stabilized for reading.

Fig. 2. Sequence diagram of analyzer. Gases are alternately drawn in and expelled as pump rocks and mercury moves from one chamber to the other. Cycle is automatically repeated to wash out residual gases.

Results

The response to carbon dioxide–oxygen mixtures was essentially complete 10 seconds after introduction of the sample into the analyzer (Fig. 3). In shifting from one mixture to another, repeated analyses were identical (within 0.1 vol. per cent) after a preliminary "washout" cycle (Fig. 4). The response to carbon dioxide of varying concentrations was linear (Fig. 5).

The anesthetic gases and vapors, mixed with air or oxygen, all produced positive readings of varying magnitude, greatest with diethyl ether, and smallest for nitrous oxide, Fluothane (halothane), and cyclopropane. The positive readings became smaller on repeated determinations (Fig. 6).

The response to mixtures of carbon dioxide, air (or oxygen), and nitrous oxide, cyclopropane, or Fluothane in anesthetic concentrations fell within 0.2 per cent of the carbon dioxide–oxygen calibration curve for 10 seconds, at which time carbon dioxide adsorption was complete. Beyond 10 seconds, there were

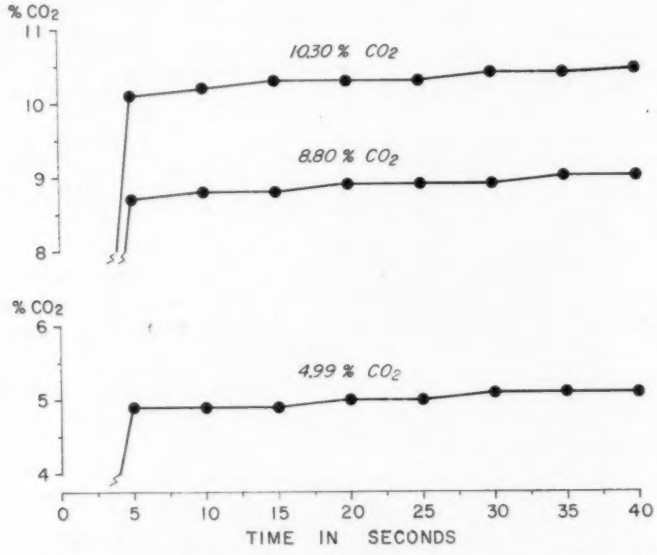

Fig. 3. Calibration of automatic analyzer with known mixtures of carbon dioxide and oxygen. Readings taken at 5 second intervals.

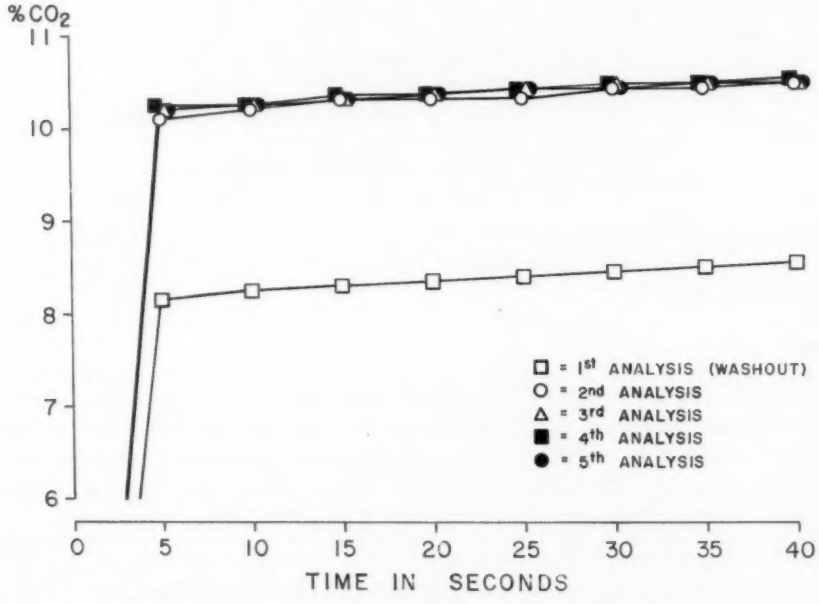

Fig. 4. Serial measurements of carbon dioxide of known (10.3 per cent) concentration demonstrating need for preliminary "washout."

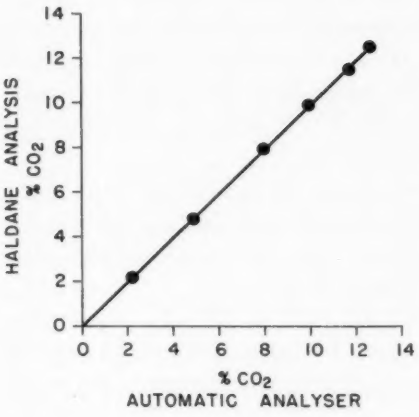

Fig. 5. Automatic analyzer measurement of carbon dioxide in known carbon dioxide-oxygen mixtures showing linear relationship. Readings were made 10 seconds after introduction of sample into analyzer. Preliminary "washout" discarded.

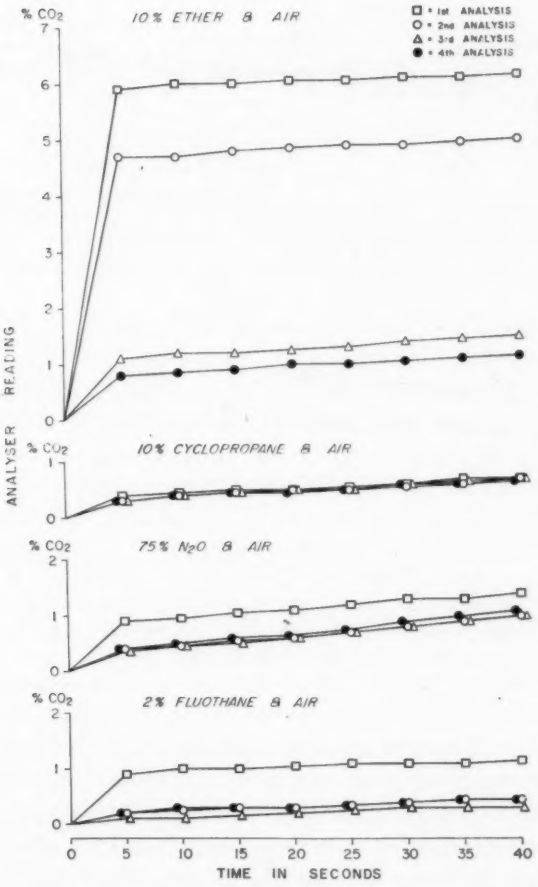

Fig. 6. False positive readings with mixtures of inhalation anesthetics and air. No carbon dioxide present.

continuous moderate to marked rises with all 3 anesthetic agents (Fig. 7). Mixtures containing diethyl ether in concentrations of less than 10 per cent behaved in a similar manner, but above 10 per cent ether concentration there was a positive error of 0.3 to 0.7 per cent present at 10 seconds and 2 "washouts" were necessary, which fact is, presumably, a reflection of the relatively slow saturation of soda lime with this anesthetic (Fig. 8).

Carbon dioxide–oxygen mixtures were found to be measured with equal accuracy when dry or moist. Dry mixtures of 8 or 10 per cent carbon dioxide in oxygen, previously checked by Haldene analysis, gave identical readings after saturation with water vapor at 25°, 37°, and 40° C.*

Rapid changes in room temperature of 10° C. (15° to 25° C.) resulted in no change in accuracy of measurement of carbon dioxide–oxygen mixtures. When cooled to 4° C. the instrument no longer was able to function. When the instrument was rewarmed, correct performance was not re-established until the machine had reached room temperature (24° C.).

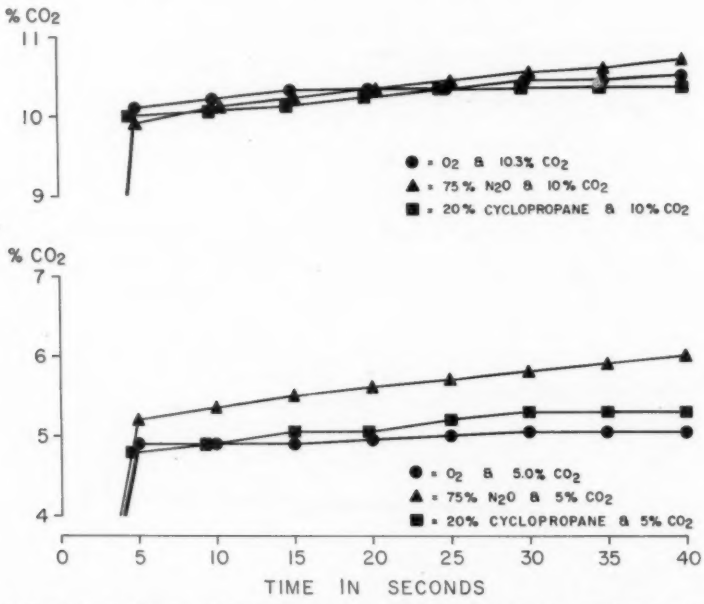

Fig. 7. Automatic analyzer measurement of carbon dioxide in mixtures of carbon dioxide, anesthetic, and air. Single preliminary "washout" discarded. At time of 10 seconds' reading, error introduced by anesthetic agent is negligible.

Comment

The rapid reaction of carbon dioxide and soda lime, and the relatively slow adsorption of anesthetic gases and vapors by the soda lime, allow to a large extent the separation of the 2 effects. By reading at 10 seconds, at which time carbon dioxide disappearance is essentially complete and adsorption of anesthetic has just begun, the error introduced by anesthetic gas or vapor should

*The instrument is calibrated for dry mixtures, and, therefore, in calculation of carbon dioxide tension in expired air, atmospheric pressure should be corrected for vapor pressure.

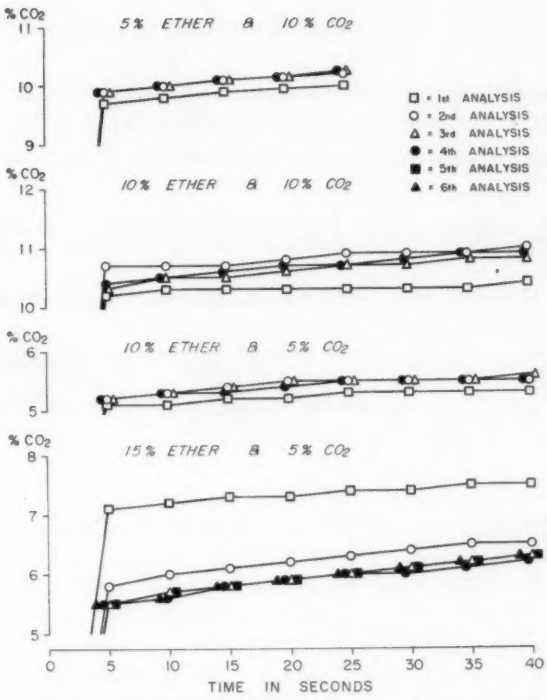
% CO2
5% ETHER & 10% CO2
□ = 1st ANALYSIS
○ = 2nd ANALYSIS
△ = 3rd ANALYSIS
● = 4th ANALYSIS
■ = 5th ANALYSIS
▲ = 6th ANALYSIS
10% ETHER & 10% CO2
10% ETHER & 5% CO2
15% ETHER & 5% CO2
TIME IN SECONDS

Fig. 8. Serial measurements of mixtures of carbon dioxide, air, and ether demonstrating increasing error with increasing anesthetic concentration and the need for 2 preliminary "washouts" with higher concentrations of this anesthetic agent.

be minimized. Surprisingly, the anesthetic introduced even less than the predicted error. Only high concentrations of diethyl ether were associated with greater errors than were observed with carbon dioxide–oxygen mixtures without anesthetic. A possible explanation for this may be that the heat of the reaction of carbon dioxide and soda lime momentarily delays the adsorption of anesthetic onto the soda lime.* Thus, the instrument presents a unique advantage for carbon dioxide analysis in the presence of anesthetic gases or vapors. The Haldane, Scholander,[2] and other analytic methods which depend on volume change following adsorption of carbon dioxide by soda lime are designed for reading after a state of equilibrium is reached, and, accordingly, allow large errors in the presence of anesthetic gases.

The instrument is suitable for use in measurement of carbon dioxide at any point within the anesthetic system; for example, it provides a convenient method for testing the efficiency of carbon dioxide removal by soda lime and the efficiency of valves. For test of adequacy of ventilation, the analyzer can be used with end expiratory or "alveolar" samples; the difficulties of alveolar sampling are great, but the estimation of arterial carbon dioxide tension by the rebreathing method of Collier and associates[3, 4] provides a simple and accurate

*The temperature within the soda lime cartridge was measured by a thermister needle, and rose approximately 3° C. within 5 seconds after injection of 50 ml. of 5 or 10 per cent carbon dioxide. The temperature elevation on the soda lime surface itself was presumably somewhat greater.

alternative which has been used extensively and with considerable success in this laboratory.

The authors suggest that the instrument is equally suitable for use outside the operating room. It has the very great advantage of simplicity and accuracy to recommend it for use for measurement of carbon dioxide in any situation.

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Quantitative determination of the serum globulins $beta_{2A}$ and $beta_{2M}$ by immunoelectrophoretic analysis

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Microimmunoelectrophoretic analysis has been used to detect consumption of antibody to β_{2A} and β_{2M} as increasing amounts of serum are added to a series of tubes containing constant quantities of antisera. By reacting the supernatants from such tubes against serum electrophoresed in agar, the volume of serum addition which results in nearly complete consumption of antibody is determined. From this end point volume, the concentration of the protein in serum can be calculated, assuming a value for the antigen equivalence of the antiserum. The method is reproducible, rapid, and relatively economical of antiserum. The determination is not affected by the presence of antibody to other proteins in the antiserum except that to gamma globulin; cross reaction between antibody to gamma globulin and β_{2M} would have an effect on the end point during assay of certain abnormal sera. The extent of the error would amount to approximately 8 per cent. No cross reaction could be detected between antibody to gamma globulin and β_{2A}.

In normal individuals, both β_{2A} and β_{2M} were found to vary widely in concentration. Values in children aged 6 months to 9 years averaged 38 per cent of the average adult level for β_{2A} and nearly equaled the adult level of β_{2M}.

The technique of immunoelectrophoretic analysis, recognized to be of value in qualitative delineation of the antigenically separate protein fractions of serum, has not been used for quantitation of protein. Only by observation of the characteristics of the precipitin lines as regards their size, shape, definition, and their distance from the electrophoretic axis is a rough estimate of the quantity of antigen possible. For the study of the several proteins which can be defined only by the immunoelectrophoretic technique, a more reliable method of quantitation would be desirable. The present report concerns details of a

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method of quantitation in which the equivalence point of the reaction of a single serum protein with its homologous antibody is determined by reacting the supernatant against serum electrophoresed in agar to detect residual antibody. The presence of reactions of other serum proteins with their antibodies occurring simultaneously does not affect the method, since the determination of specific residual antibody is made possible by the immunoelectrophoretic distribution. With the use of the micro-,[1] as opposed to the macroimmunoelectrophoretic technique, the results are reproducible, relatively small quantities of serum and antiserum are necessary, and determinations can be made relatively quickly. The present paper describes the method as it is applied to the two β_2 globulins, β_{2A} and β_{2M}.

Because these β_2 globulins were not available in pure form, their concentrations have been expressed in arbitrary units derived in terms of the standard antiserum used. The unit has been defined as the amount of the protein which will be precipitated by the antibody contained in 1 ml. of the standard antiserum. For the quantitation, increasing volumes of the unknown serum are added to a series of tubes containing a constant volume of antiserum, and, after incubation, the supernatants of the mixtures are tested for specific antibody by reacting them against a standard pooled serum electrophoresed in agar. The end point value is the volume of serum addition which results in a supernatant giving a very faint precipitin line for the specific protein, indicating nearly complete removal of the specific antibody. The volume of serum necessary to add to 1 ml. of the standard antiserum to reach the end point is then considered to contain one unit of the protein in question.

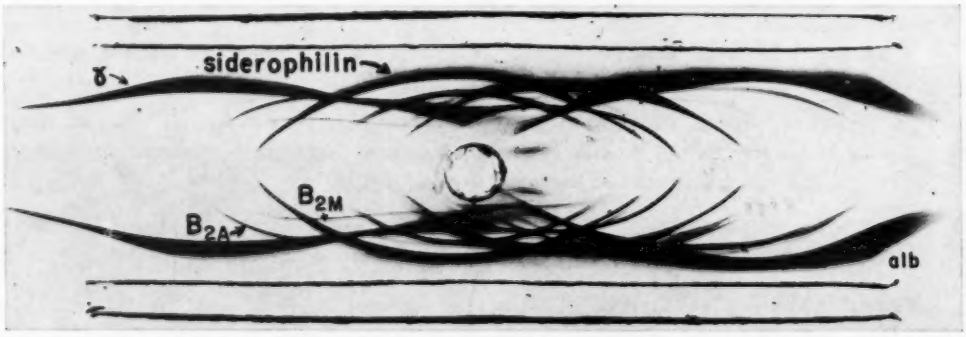

Fig. 1. Immunoelectrophoretic pattern resulting when the standard antiserum, used for quantitation in this study, is reacted with normal human serum.

Materials and methods

The standard antiserum was produced in a 1-year-old goat by injection of whole human serum incorporated in Freund's adjuvant. The adjuvant was made in the usual 8.5:1.5:10 mixture. Serum was diluted 1:6 in saline before incorporation in adjuvant. Subcutaneous injections of approximately 12 ml. of adjuvant were given on 6 occasions, 1 week to 10 days apart. Each dose was given as multiple injections of 1 to 2 ml. Two months after completion of this initial series, a booster injection of 10 ml. of adjuvant was given. Antiserum was obtained on several occasions thereafter by withdrawal of 150 to 200 ml. of blood. This antiserum gave well defined lines representing β_{2A}, β_{2M}, and other serum proteins when it was reacted against normal adult serum (Fig. 1). Antiserum was preserved

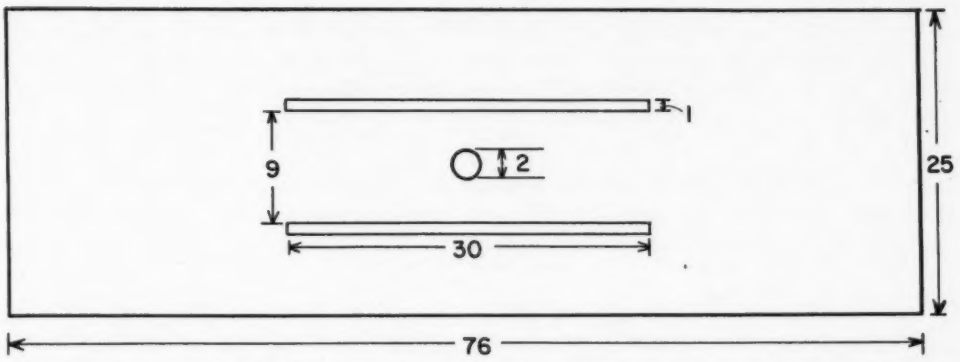

Fig. 2. Pattern of the antibody troughs and antigen well used for immunoelectrophoresis. Dimensions are in millimeters.

by storage at –10° C.; repeated freezing and thawing of antiserum did not detectably affect its potency.

The microimmunoelectrophoretic method used employed standard glass microscope slides as originally described by Scheidegger.[1] A 2.5 ml. aliquot of melted 2 per cent agar in veronal buffer, pH 8.2, ionic strength 0.05, was pipetted rapidly on a glass slide and allowed to solidify. A well for antigen and troughs for antibody were then cut, in the dimensions shown in Fig. 2. The antigen well was cut with a blunted 14 gauge needle and the agar was removed by suction. A template made of razor blades inserted in a plastic block was used to cut the antibody troughs and the slivers of agar were teased out with a needle. Electrophoresis was carried out in an apparatus accommodating 8 slides, adjustment of voltage and amperage having been made so that distribution of the proteins would be complete in 45 minutes.

In preparing supernatants, various volumes of unknown serum were added to 0.2 ml. aliquots of the standard antiserum. The amounts used and the corresponding values for the protein concentration at the end point, expressed in arbitrary units, are given in Table I. Serum additions were made with serologic pipettes of 0.1 ml. capacity graduated in 0.001 ml. As noted in Table I, serum was diluted fivefold prior to pipetting of small volumes. Arbitrarily, the greatest serum addition was placed at 0.1 ml. per 0.2 ml. of antiserum. For serum additions less than this, a volume was added of 0.15 M NaCl, sufficient to bring the total volume of the addition to 0.1 ml. In practice, a general range for the concentration of β_{2A} and β_{2M} was usually first estimated by the appearance of the precipitin lines representing these proteins when the serum was reacted by the standard microimmunoelectrophoretic technique. This range was then encompassed by the appropriate mixtures (Table I). The trial often had to be repeated to obtain the end point. About 2 ml. of antiserum was usually required (10 mixtures) for the determination of β_{2A} and β_{2M} in a single specimen.

After the serum-antiserum mixtures were prepared they were incubated for 30 minutes at 37° C. and then for 48 hours or more at 4° C. At the conclusion of this time, a standard pooled serum was electrophoresed in agar, as described above, and the supernatants of the serum-antiserum mixtures placed in the antibody troughs. The standard pooled serum was a mixture of equal volumes of serum from 10 healthy adults and the volume of it, electrophoresed, was always 0.005 ml., measured into the antigen well by means of a Sanz pipette.[2] After addition of supernatants, the slides were incubated overnight at room temperature in a moist chamber and the following morning examined with a magnifying glass or binocular loupe (X 2.3) on the glass top of an internally illuminated viewing box or by side lighting from a gooseneck lamp. In each case, the background was dark and light was transmitted through the slide at an angle providing for maximum definition of the precipitin lines. The end point was taken as the amount of serum which resulted in the line being visible, but faint, with further stepwise increase (Table I) in the amount of serum addition resulting

in disappearance of the line.* Extrapolation was frequently used in estimating the end point. If the precipitin line was well defined and relatively easy to see, but was absent with the next larger serum addition, the end point was extrapolated to a volume intermediate between the two. On the other hand, a supernatant producing a very faint precipitin line was considered to be at the end point.

Table I. Composition of serum-antiserum mixtures for quantitation of β_{2A} and β_{2M}

Volume of serum added to 0.2 ml. antiserum (ml.)	*Volume of serum per 1 ml. antiserum (V) (ml.)*	*Concentration of protein in serum (S)* S = 1/V units/ml.)*
Diluted 1 → 5		
0.01	0.01	100.0
0.02	0.02	50.0
0.03	0.03	33.3
0.04	0.04	25.0
0.05	0.05	20.0
0.06	0.06	16.7
0.07	0.07	14.3
0.08	0.08	12.5
0.09	0.09	11.1
0.10	0.10	10.0
Undiluted		
0.025	0.125	8.0
0.03	0.15	6.7
0.035	0.175	5.7
0.04	0.20	5.0
0.05	0.25	4.0
0.06	0.30	3.3
0.08	0.40	2.5
0.1	0.50	2.0

*Concentration of β_{2A} or β_{2M} if supernatant of mixture is at end point. Values are in terms of arbitrary units calculated on basis that 1 ml. of standard antiserum contains sufficient antibody to precipitate 1 unit of β_{2A} or β_{2M}.

The concentration of the antigen per milliliter of serum (S), expressed in arbitrary units, was calculated as $S = \frac{A}{V}$ in which A is the units of antigen which will be precipitated by the antibody contained in 1 ml. of antiserum and V is the volume of serum addition per milliliter of antiserum which results in the end point. For the standard antiserum, an arbitrary value of 1 was assigned to A for both β_{2A} and β_{2M}.

The reproducibility of the method was assessed by determining on repeated runs the end point of mixtures of antiserum and standard pooled serum. Such mixtures were initially run with each batch of unknowns.

For tests for cross reactions involving antibody to β_{2A}, β_{2M}, and gamma globulin, the antibody to gamma globulin was absorbed from the goat antiserum by 3 preparations:

*Although the end point is described as the equivalence point, it is actually on the antibody excess side of the equivalence point. The faint precipitin line taken as defining the end point may be deceptive in indicating the amount of antibody present. The amount may be relatively large, since the antiserum is always considerably diluted, not only by the addition of serum and saline (dilution of 1→1.5), but also by its diffusion through agar to reach the point of precipitation. In the method the extent of both these dilutions is maintained relatively constant.

commercially prepared Fraction II,* slow gamma globulin, and cord serum. The slow gamma was prepared by continuous flow curtain electrophoresis of the Fraction II. Only tubes at the cathodic end of the gamma band were taken, representing about one fourth to one third of the electrophoretic protein distribution. The solutions were dialyzed against distilled water and concentrated by pervaporation. The concentration of gamma globulin after dialysis was determined by absorption at 280 μ. One milligram amounts of Fraction II and of slow gamma were added per milliliter of antiserum. Cord serum was from a normal full term infant and was added in the amount of 0.1 ml. per milliliter of antiserum. After the additions, the mixtures were incubated at 37° C. for 30 minutes and at 4° C. for 48 hours.

Results

Reproducibility. An estimate of the reproducibility of the method can be obtained from the results with the supernatants of mixtures of antiserum and standard pooled serum which were repeatedly run with each batch of unknowns. The reproducibility for β_{2A} was striking in that, in 43 of 44 runs performed over a 45 day period, the line for β_{2A} was only faintly visible with additions equivalent to 0.05 ml. of normal pooled serum to one ml. of standard antiserum and was absent with additions of 0.06 ml. On one occasion a line was faintly visible after the 0.06 ml. addition. The concentration of β_{2A} in the pooled serum was, therefore, 20 units per milliliter. The β_{2M} quantitation was not so reproducible, Of 37 runs the end point was at 0.07 ml. in 3, 0.08 ml. in 22, and 0.09 ml. in 12. The concentration of β_{2M} thus could be said to lie between 14.3 and 11.1 units per milliliter; the average value was calculated as 12.1 units per milliliter.

The failure to obtain reproducible results with the β_{2M} quantitation was probably the result of failure initially to employ optimum lighting in reading the slides. Subsequent studies, reading by a binocular loupe and employing bright illumination from a gooseneck lamp, exclusively, have shown the β_{2M} end point to be as reproducible as that for β_{2A}.

Further attesting to the accuracy of the estimation of residual antibody were the results of assay of several identical series of mixtures prepared independently by different individuals. The supernatants were of sufficient volume that they could be tested for residual antibody on several occasions. The end points in a series of mixtures made by one individual were found to be highly reproducible from one electrophoretic run to another, but among the series made by the different individuals the end points varied significantly. The results indicate that there are few variables in the detection of residual antibody by the immunoelectrophoretic technique and that a limiting factor in accuracy can be the preparation of the serum-antiserum mixtures.

Effect of varying concentration of reactants on end point. In reactions in agar, as in aqueous media, the amount of precipitate formed is dependent on the relative concentrations of antigen and antibody. In the quantitation procedure described, the concentration of antibody diminishes steadily as the end point is approached, whereas the concentration of antigen remains constant at the level present in the normal pooled serum. It is easily possible that, at the low antibody concentration present at the end point, an excess of antigen would cause the precipitate to dissolve and perhaps constitute a variable in the method.

*Pentex, Inc., Kankakee, Ill.

Experiments designed to investigate the effects of various concentrations of antigen on the end point indicate that relatively low concentrations enhance the visibility of the precipitin line. Thus when serum specimens with concentrations of β_{2A} varying from 5 to 100 units per milliliter, electrophoresed in agar, were reacted with supernatants of very low antibody concentration, the β_{2A} line was not seen in sera with the highest concentration of this protein but became visible at lower concentrations and was most distinct when the concentration was 5 units.

Similar studies with sera with β_{2M} concentrations encompassing a wide range did not show the same trend as was seen with β_{2A}. Results similar to those for β_{2A} were seen however, when serial dilutions of a single serum with a high β_{2M} concentration were electrophoresed. With a supernatant of low antibody concentration, the β_{2M} line was absent at β_{2M} concentrations of 33 and 22 units per milliliter, became visible and sharper at concentrations of 17 and 10 units, and disappeared again at 5 units.

These results indicate that minor variations in the concentration of the protein in the electrophoresed serum will have little effect on the end point, but that large variations will affect it. The concentration of 20 units per milliliter for β_{2A} and of 12.1 units for β_{2M} in the pooled serum used may be slightly high for maximum definition of the end point. It is possible that, for β_{2A} at least, slightly greater precision and sensitivity might be obtained by reacting the supernatants against pooled adult serum diluted with an equal volume of saline. The lack of effect of minor variations in protein concentration on the end point also suggests that electrophoresis of an exact amount of pooled serum (in this study, 0.005 ml.) is not necessary. When the slides are carefully made, the antigen well probably has a volume which is sufficiently constant and reproducible to result in a reproducible end point.

Cross reactions involving β_{2A}, β_{2M}, and gamma globulin and their effect on quantitation. Cross reactions of such a nature that antibody reacts both with β_{2A} or β_{2M} on the one hand and with gamma globulin on the other would result, if extensive, in an error of major magnitude in the present method. Such a cross reaction between antibody to gamma globulin and β_{2M} has been described by Franklin and Kunkel,[3] and Heremans and associates[4] have suggested that antigamma may also cross react with β_{2A}. The effect of such cross reactions on the quantitation would be to reduce the volume of serum required for the end point, thus giving a falsely high concentration of the protein in terms of units per milliliter. The amount of the error would be related to the concentration of antibody to gamma globulin in the supernatant, a factor in turn dependent on the antigamma titer of the antiserum and on the amount of gamma globulin in the serum being tested.

It should be noted that the cross reaction would be operative only at the stage where serum is added to antiserum; cross reactions would not occur in the agar, since antibody-to-gamma precipitates before it can reach the β_{2A} or β_{2M} of the electrophoresed serum.

To determine the presence of such cross reactions and the magnitude of the error which might result, serum was added in increasing amounts to a series of tubes containing constant quantities of antiserum from which the antibody to gamma globulin had been absorbed. The end point was compared with that for similarly treated, unabsorbed antiserum to which a volume of saline had been added equal to that of the absorbing solution. In all cases, antibody to gamma globulin was present in abundance in the end point supernatants from unabsorbed antisera.

In comparing the end points with the absorbed and unabsorbed antisera it should be noted that two factors could be responsible if the volume of serum at the end point is greater in the unabsorbed than in the absorbed serum. Cross reactions could be occurring, with antibody-to-gamma globulin reacting with common reactive sites on β_{2A} or β_{2M}, in which case more β_{2A} or β_{2M} would need to be added to the unabsorbed serum before antibody specific for these proteins would be depleted. Alternatively, by contamination of the absorbing material with β_{2A} or β_{2M}, the titer of antibody for these proteins in the absorbed antisera could be reduced, again allowing more of the proteins to be added to the unabsorbed sera before the end point is reached. The contrary result, a lower volume of serum required for the end point with the unabsorbed antisera, could only be the result of a technical error. Equality of end points would of course mean absence of cross reactions and absence of contamination of the absorbing material.

The results with absorbed and unabsorbed antisera in the case of β_{2A} are shown in Fig. 3. The first two boxes represent the results after absorbing the antiserum with commercially available Fraction II and show a considerable difference in end point in a direction compatible with marked contamination of the Fraction II with β_{2A}. Testing of the Fraction II for β_{2A} indicated that,

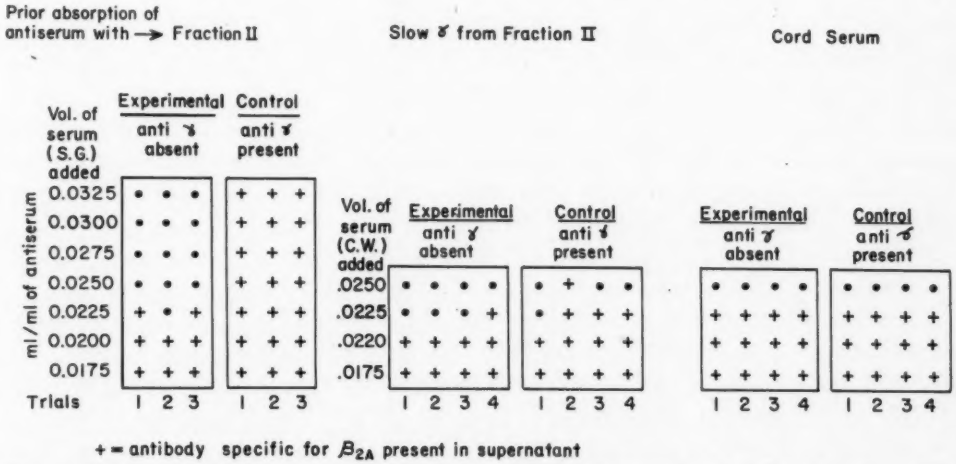

Fig. 3. Effect of absorption of antibody to gamma globulin on end point for β_{2A}. The end points of antisera from which gamma has been absorbed by addition of Fraction II, slow gamma from Fraction II, and cord serum (experimental) are compared with end points of the same antisera not absorbed (control).

Table II. Serum concentrations of β_{2A} and β_{2M} in normal children and adults

Subject	Age		Sex	β_{2A} concentration		β_{2M} concentration	
	Years	Months		Units per ml.	Per cent of average adult value*	Units per ml.	Per cent of average adult value*
Children.							
M. R.		8	M	8	40	15	125
G. B.		8	M	4.5	22	5.5	46
C. B.		10	F	11	55	11	92
M. U.		10	F	8	40	8	67
Y. S.	1		F	11	55	15.5	129
C. S.	1		F	4	20	6	50
A. M.	1		M	8	40	18.5	154
C. S.	1	2	F	6	30	13.5	112
J. S.	1	2	F	6.5	32	18.5	154
J. P.	1	4	M	4.5	22	5.5	46
D. D.	1	6	F	7.5	37	13.5	112
M. M.	1	6	F	4.5	22	5.5	46
R. H.	1	7	F	8	40	7	58
L. R.	2		M	10	50	10	83
L. O.	2	8	F	5	25	12.5	104
J. F.	2	11	F	8	40	15.5	129
G. G.	3		M	8	40	5	42
A. M.	4		F	10	50	8	67
J. V.	5	6	M	11	55	9	75
C. L.	9		F	11	55	11	92
Averages				7.7	38.5	10.7	89.1
Adults.							
C. W.			M	29	145	11	92
W. B.			M	18.5	92	6.5	54
W. S.			M	18	90	9	75
V. H.			M	25	125	5	42
R. Z.			M	25	125	16.5	137
G. C.			M	14.5	72	16.5	137
A. M.			M	29	145	8	67
F. L.			M	16.5	82	20	167
J. H.			M	16.5	82	8	67
R. H.			M	29	145	8	67
N. H.			F	13.5	67	13.5	112
L. H.			F	25	125	12.5	104
R. M.			F	13.5	67	14.5	121
M. L.			F	12.5	62	11	92
J. B.			F	14.5	72	9	75
N. P.			F	22.5	112	14.5	121
W. G.			F	20	100	15.5	129
H. P.			F	20	100	13.5	112
C. G.			F	16.5	82	17.5	146
V. F.			F	12.5	62	12	100
Averages				19.6	100	12.1	100
Values for standard pooled serum (10 adults)†				20		12.1	

*Values calculated on basis of mean adult concentration equal to 100. For calculation, mean concentration of β_{2A} taken as 20 and of β_{2M}, 12 units per milliliter.

†Averages of 44 determinations of β_{2A} and 38 determinations of β_{2M} on a single pooled serum (see text).

indeed, 0.15 to 0.3 units of β_{2A} was present per milligram of material, enough to precipitate all the antibody for this protein in 0.25 to 0.5 ml. of the antiserum used in these experiments. The use for absorption of only the slow gamma portion of this fraction, obtained by curtain electrophoresis, almost eliminated the discrepancy in end points, as can be seen in the second pair of boxes in Fig. 3. It is probable that the small discrepancy remaining is again the result of slight contamination with β_{2A}, since absorption of antiserum with cord serum, as shown in the last pair of boxes, gave complete agreement in end point in absorbed and unabsorbed antisera. One can conclude that there is little, if any, precipitation of β_{2A} by antibody to gamma globulin and that β_{2A} is absent from the cord serum employed.

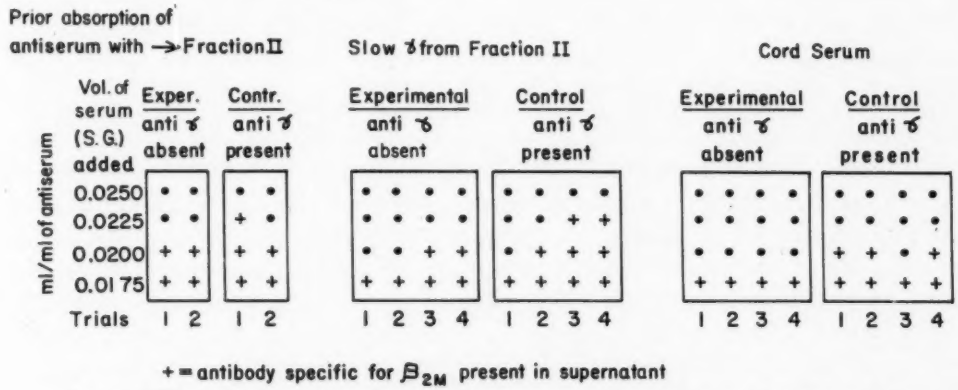
Prior absorption of antiserum with → Fraction II
Slow γ from Fraction II
Cord Serum
Vol. of serum (S.G.) added
ml/ml of antiserum
Exper. anti γ absent
Contr. anti γ present
Experimental anti γ absent
Control anti γ present
Experimental anti γ absent
Control anti γ present
0.0250
0.0225
0.0200
0.0175
Trials 1 2 1 2 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
+ = antibody specific for β2M present in supernatant

Fig. 4. Effect of absorption of antibody to gamma globulin on end point for β_{2M}.

The results with β_{2M} were more consistent (Fig. 4). With all three methods of absorbing gamma globulin the unabsorbed antiserum required, on the average, slightly more serum for the end point than the absorbed. In a total of ten trials, end points were the same in only three. Combining all the trials, an average of 8 per cent more serum was needed to reach the end point with unabsorbed antiserum. It cannot be stated categorically that this discrepancy is the result of a cross reaction, since it could equally well be explained by contamination of all the absorbing materials with β_{2M} to a similar extent. Cord serum is known to contain small amounts of β_{2M}.[5] Whether contamination or a cross reaction is responsible, the difference in end points is of such small magnitude that it falls within the inherent error of the method. With the antisera employed in the present study the error from cross reaction would not occur except in titering sera with very low gamma or high β_{2M} concentration, since, normally, antibody to gamma globulin was absent at the end point.

Serum concentrations of β_{2A} and β_{2M} in normal subjects. Values for β_{2A} and β_{2M} in the serum of 20 children and 20 adults are given in Table II. Considerable range of variation is apparent for both proteins. For β_{2A}, in adults, the variation from the mean amounted to ± 40 per cent. Although the levels in the men tended to be higher than in the women, averaging 22.1 versus 17.0, the difference was not of a high level of significance statistically ($p = .05$). Of interest was the fact that children had an average level of β_{2A}

amounting to only 38 per cent of the average adult value. Again, among the children, there was wide variation in level, but none of the values were as high as the lowest adult values. In this series, there was no clear indication of increasing values with increasing age; the average level of the 10 children of ages 6 months to 1½ years was 7.1, and of the 10 aged from 1½ to 9 years, 8.3, a difference which was not significant statistically ($p = .3$).

The values for β_{2M} also varied widely. Deviations amounting to ± 60 per cent of the mean value were relatively frequent. Again there was a slight difference in average values for males and females, 10.8 and 13.3, respectively, but this was not significant ($p = > .1$). In contradistinction to β_{2A}, levels of β_{2M} were not significantly lower in the children than in the adults. Again there was no evidence of the level increasing with age.

Discussion

Because of their implied or apparent relation to the immune system of the body, the proteins designated β_{2A} and β_{2M} together with gamma globulin, have been termed the immunoglobulins.[6] Evidence for their association with the immune system comes from several observations. All three proteins are greatly reduced in amount or absent from the serum of agammaglobulinemics.[7] β_{2A} and β_{2M} are in low concentration or absent in the newborn period and in certain patients predisposed to infection.[8-10] One of the latter patients was unusually tolerant to a homotransplant of skin.[9] In addition, as is well known, several antibodies are known to be macroglobulins and to reside in the fraction known by immunoelectrophoretic analysis as β_{2M}.

Methods for the quantitative determination of these globulins, described elsewhere,[6] are cumbersome and unsatisfactory. β_{2M} has been quantitated by the use of the ultracentrifuge and found in normal serum to constitute less than 2 per cent of the total protein.[11] Quantitation of β_{2A} has not been possible. It would appear that the method herein described, although not yielding absolute values, might give information of interest when applied to the study of patients with immunologic defects.

Several theoretical and practical disadvantages of the method are apparent. In the category of a theoretical disadvantage is the error which may result from reactions of a given antibody with groupings on the 7 S gamma globulin which are also present on either β_{2A} or β_{2M}. Evidence for such a cross reaction involving β_{2A} is only suggestive[4] and could not be demonstrated in the present study. It is not considered necessary in the present paper to review the rather large volume of literature bearing on the question of the cross reaction between β_{2M} and antibody to gamma globulin. In brief, the evidence for such a cross reaction derives from two sources. One is the demonstration by the agar double diffusion technique that pathologic macroglobulins of the 19 S type have antigenic groupings in common with groupings on both 7 S gamma globulin and on the normal 19 S macroglobulin (β_{2M}).[12, 13] Although these interrelationships suggest that antibody made to gamma globulin would react also with 19 S macroglobulin, technical difficulties prevented demonstration of this by the agar gel technique.[3] The second line of evidence arises from the

results of studies by Franklin and Kunkel[3] using the quantitative precipitin technique. These studies indicated that the β_{2M} and gamma globulins partially cross react and are antigenically related. The present studies can be similarly interpreted, but, within the rough quantitation permitted by them, the extent of the cross reaction would appear to be considerably less than indicated by the precipitin curves of Franklin and Kunkel. This discrepancy in degree may reflect a difference in the groupings against which goat antibody is directed as compared to the sites reactive to the rabbit antibody used by them.

A practical disadvantage is that, in the absence of a pure preparation of the protein, the method can be standardized only in terms of a standard antiserum. The best means at present to prepare a standard antiserum would appear to be by the use of a pooled serum consisting of equal amounts of serum from 10 to 20 healthy adult individuals equally divided as to sex. Assuming this serum pool to have a β_{2A} or β_{2M} concentration (S) equal to the average for adults found in the present paper, the end point volumes for the serum pool (V) can be substituted in the equation $S = \frac{A}{V}$ and a value for the titer of the antiserum (A) determined. This value is the units of protein precipitated by the antibody contained in 1 ml. of the antiserum and would henceforth be used for calculation of concentration in the unknowns.

The levels of β_{2A} and β_{2M} in normal individuals are of interest in several respects. As indicated by the study of Hitzig,[8] precipitin lines representing β_{2A} and β_{2M} are absent in the sera of infants up to the age of 2 to 3 months, and in some infants the lines cannot be seen at the age of 1 year. From the present study it would appear that all during childhood the level of β_{2A} is relatively low. It will be of interest to see whether a marked increase to adult values occurs at adolescence. With our antisera, standard microimmunoelectrophoresis will not reveal the β_{2A} line if the level is 6 units per milliliter or lower. From Table II it is apparent that a number of normal children under the age of 3 would, therefore, have no visible β_{2A} line, yet, presumably, have normal levels of this protein. The values give meaning to the general observation that the β_{2A} line in the serum of children is usually fainter than in the sera of adults. In contrast to β_{2A} the levels of β_{2M} although also very low in infancy, as noted by Hitzig,[8] very quickly reach levels close to those of the adult. The rate of production of this protein in the infant and child would appear to parallel that of gamma globulin.

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An ion exchange method for plasma ammonia concentration

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A full description of an accurate method for determining plasma ammonium concentration is given. The ammonium in 3.0 ml. of plasma is adsorbed on 2.7 Gm. of cation exchange resin. The resins are washed and the ammonium eluted directly into Nessler's solution with sodium hydroxide. Spectrophotometer readings of the solution are converted to ammonium concentration by reference to a standard curve. The reproducibility of the method is given. Possible sources of error are discussed.

The method is used to demonstrate the changing rate of alkaline hydrolysis of blood plasma to ammonia. These data are used to explain the low normal values obtained by this method in comparison to those obtained by diffusion techniques.

The unsatisfactory correlation of blood ammonium concentrations with clinical symptoms in liver disease may be due in part to inadequacies in the analytical procedures.[1-3] The methods of Conway,[5] Seligson,[6] and subsequent modifications[7, 8] involve alkalization of the blood or plasma to convert ammonium into the diffusible gas ammonia. The danger of falsely high values for ammonia due to alkaline hydrolysis of amide groups in proteins and amino acids is well recognized,[7] and data are presented here which demonstrate this error. An ion exchange method is described in which the blood or plasma is not subjected to such ammonia forming pH changes.

Materials

The following is a list of the materials necessary for this ion exchange method.

1. Strongly acidic cation exchange resin (Dowex 50W– × 16).†
2. Mixed bed resin (Amberlite MB3).‡

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†J. T. Baker Chemical Co., Phillipsburg, N. J.
‡Rohm and Haas Co., Philadelphia 5, Pa.

3. Nessler's stock solution[9] (diluted 1:10).

4. Sodium hydroxide 0.1 N.

5. Resin processed wash water (distilled water passed through mixed bed resin column).

6. $KNaHPO_4/KH_2PO_4$ buffer (pH 7.40) (0.2 Molar – PO_4) prepared with resin processed wash water.

7. Ammonium standard solution containing 0.1 Gm. of ammonium nitrogen (as NH_4Cl) per liter (resin processed wash water).

8. Four to 5 Gm. of ghatti gum crystals in gauze which have been left in 75 ml. of distilled water overnight to form a solution. A few thymol crystals are added to inhibit bacterial growth.

9. A Coleman Model 14 spectrophotometer. Two pairs of Coleman cuvettes (14 by 105 mm. round, Order No. 14-303) and holder (Order No. 14-100).

10. pH meter (for titrating buffer).

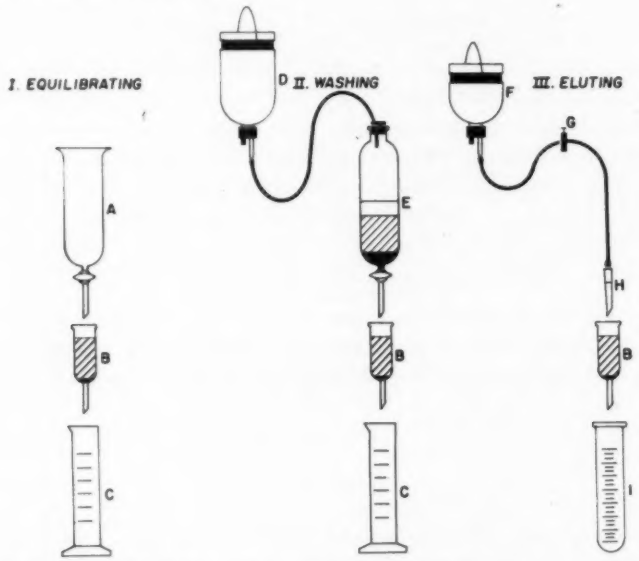

Fig. 1. Diagrammatic representation of apparatus employed in the method. Apparatus includes: *A*, reservoir; *B*, glass ammonium adsorption column; *C*, 100 ml. graduate; *D*, a one liter intravenous feeding bottle; *E*, mixed bed resin column; *F*, 500 ml. intravenous feeding bottle; *G*, screw clamp; *H*, tapered glass tube; and *I*, graduated test tube.

The apparatus necessary for this procedure are listed as follows:

A. Five 100 ml. glass reservoirs with stopcocks.

B. A dozen glass ammonium adsorption columns. The glass portion of discarded Venopak* sets may be used. They are premarked at 5/8 inch from the top to contain approximately 2.7 Gm. of wet cation exchange resin. Fresh resins are used for each determination. A fine wisp of glass wool is placed inside against the beveled end to retain the resins.

C. Six 100 ml. graduated cylinders.

D. A one liter intravenous feeding bottle and tubing.

E. Mixed bed resin column. It is convenient to use separate mixed bed columns for supplying wash water during the procedure and for making up the buffer. Cylindrical glass separatory funnels (100 ml.) fitted with plastic screen or glass wool to retain resins are used.

F. Five hundred milliliter intravenous feeding bottle for 0.1 N NaOH. The small piece of alloy metal in the outflow side of the screw-cap should be removed.

G. Screw clamp or stopcock to regulate flow of NaOH.

*Abbott Laboratories, Needham Heights, Mass.

H. Small glass tube finely tapered on one end.
I. Two dozen 25 ml. graduated test tubes.

Methods

Preparation. Calibrated test tubes are washed in detergent solution, rinsed, soaked in 0.1 N NaOH, and removed to racks for drying before use. Ammonium adsorption columns *(B)* with glass wool in place are filled to the mark with fresh resins by means of a small funnel and wash bottle. Glass reservoirs *(A)* are rinsed twice with 20 to 30 ml. of buffer and finally filled with 100 ml. of buffer.

Equilibrating resins. Thirty milliliters of 0.1 N NaOH are dripped from one reservoir *(A)* through each ammonium adsorption column *(B)* (10 minutes). Then 100 ml. of pH 7.40 buffer is dripped from another reservoir *(A)* through each column (20 to 25 minutes). Several columns may be equilibrated simultaneously.

Obtaining blood samples and preparation of plasma. Blood samples are drawn without stasis in heparinized syringes (heparin filling the dead space). We, as others,[4] have found that some brands of heparin contain significant concentrations of ammonium. We currently use heparin from The Vitarine Company, New York, N. Y. The blood is brought directly to the laboratory and centrifuged in test tubes. Samples of whole blood may be left at least an hour without change in the ammonia concentration.

Washing columns and adsorption of ammonium from plasma. An equilibrated ammonium adsorption column *(B)* is placed under the mixed bed resin column *(E)* and 10 ml. of resin processed wash water run through the column in one to 2 minutes. Three milliliters of plasma are now pipetted slowly (1½ minutes) into the top of a washed adsorption column directly onto the resin. Next, 40 ml. of resin processed wash water are passed through the column. This column is now ready for eluting but is stable for at least 12 hours, or until resins begin to dry. Columns are collected and eluted at the end of the day.

Preparation of standard curve. One, 2, and 3 ml. of the ammonium standard solution are added to 100 ml. volumetric flasks. These are filled to volume with resin processed wash water, thus they contain 1, 2, and 3 μg per milliliter of ammonium nitrogen, respectively (or 71, 142, and 223 μEq per liter).

For a blank, an ammonium adsorption column is washed with 50 ml. of resin processed wash water. For a standard, a 3.0 ml. pipette is rinsed with the standard and then used to pipette 3.0 ml. of standard slowly (1½ minutes) into an equilibrated and washed adsorption column. This is followed by 40 ml. of wash water.

Eluting samples and reading. The sensitivity of response of the colorimeter should be adjusted each day using a standard wavelength filter. Two cuvettes are filled with water and read against each other. Any difference should be noted and later added or subtracted from the final reading. One cuvette remains filled with water and is used as a reference. All samples are read in the other cuvette at 405 mμ. The sample cuvette is rinsed thoroughly with wash water between samples.

The ammonium is eluted from the ammonium adsorption column *(B)* by dripping 0.1 N NaOH *(F)* from the tapered glass tube *(H)* through the adsorption column into a graduated test tube *(I)* containing one drop of ghatti gum solution. This is run at 70 to 90 drops per minute until the tube is filled to the 9.0 ml. mark. One milliliter of Nessler's reagent is added immediately and the sample mixed by gentle swirling. The solution is transferred to the sample cuvette and read against the reference at 5 minutes. The reference cuvette must read exactly zero before and *after* the sample.

A standard curve is drawn by plotting optical density readings of the samples against μg per milliliter of the standard solution. Plasma samples are read from this curve.

Results

The standard curve of ammonium recovery from cation resin is shown in Fig. 2 and compared to the addition of Nessler's reagent to ammonium standards

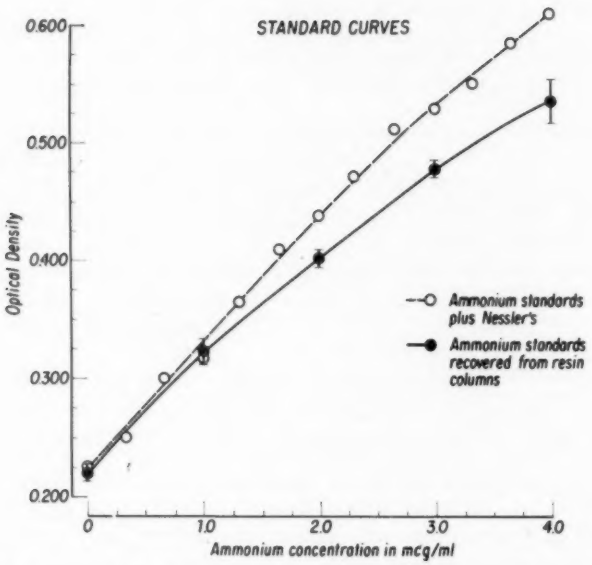

Fig. 2. All points are obtained by plotting optical density readings of ammonia–Nessler's solutions against ammonium concentrations. Each point is fixed by 10 consecutive plots. One standard deviation from the mean on each side of the line is indicated. This line is used to read plasma samples.

directly. The width of one standard deviation on each side of the line is shown. This standard deviation expressed as ammonium nitrogen is 0.09 to 0.12 μg per milliliter for the blank and the one, 2, and 3 μg per milliliter standards. This deviation increases to 0.3 μg per milliliter with the 4.0 μg per milliliter standard.

Ammonium concentration in plasma is stable for over an hour at room temperature. Fresh human plasma measured 0.48, 0.42, and 0.50 μg per milliliter at 20 minutes and one and 2 hours, respectively. Duplicate plasma samples agree closely. Seven determinations on the same plasma had a standard deviation of 0.03 μg per milliliter.

The possible loss of ammonia from plasma in a test tube during centrifugation was evaluated as follows: three samples at different concentrations were each divided into 2 portions. One portion was left in the syringe and centrifuged. This plasma was transferred air-free from the syringe to a pipette and processed for ammonia. The second portion was centrifuged in a test tube exposed to air. Duplicate determinations of these portions showed no difference in ammonia concentrations whether handled in syringes or in air. This evidence, together with the observation that plasma ammonium concentration is constant in test tubes for an hour after centrifuging, have led us to handle blood samples in test tubes.

Ammonium standards added to plasma give the same slope as the standard curve. The addition of sodium, potassium, and chloride to standards in concentration equal to plasma does not change the slope of the standard curve.

Venous blood for 18 normal individuals varied from 0.0 to 0.42 μg per

milliliter of ammonium nitrogen with an average of 0.25 μg per milliliter. In practice the values are only read to the nearest one tenth microgram.

Because of the discrepancy between normal values for diffusion techniques[5-8] and the ion exchange method, a study of the rate of ammonia formation from alkalinized plasma was carried out as follows: three milliliter samples of dog plasma were alkalinized air-free in syringes (pH 11.7) with 1.0 ml. of 0.22 N KOH. Consecutive samples were allowed to stand for increasing intervals of time before neutralizing with 1.0 ml. of 0.2 N H_2SO_4. Controls were run by the addition of 2.0 ml. of the combined acid and base. Three milliliters of the final mixture were run through resin columns and the ammonia determined. The results are shown in Fig. 3.

In some diffusion methods[4] the rate of ammonia formation from alkalinized plasma is assumed to be constant. Fig. 3 indicates an accelerated production of ammonia during the first 5 minutes. A method extrapolating back to zero time using only the rate of increase of ammonia concentration after 5 minutes would find a value of 2.05 μg per milliliter. Extrapolating back to zero time on the control line gives a value of 1.65 μg per milliliter. This is 0.4 μg per milliliter lower. This value corresponds to the difference of normal values between these diffusion methods and ion exchange.

The problem of possible protein contamination and hydrolysis is present in the ion exchange method. At pH 7.40 a minute amount of relatively electropositive protein is adsorbed from the plasma and later eluted. This can be demonstrated by passing the eluent directly into concentrated trichloracetic acid and observing a faint precipitate.

The possible contribution of ammonia from this protein has been evaluated in the following way: free ammonia standard reacting with Nessler's reagent produces most of its color in 3 to 4 minutes. A subsequent slow maturation

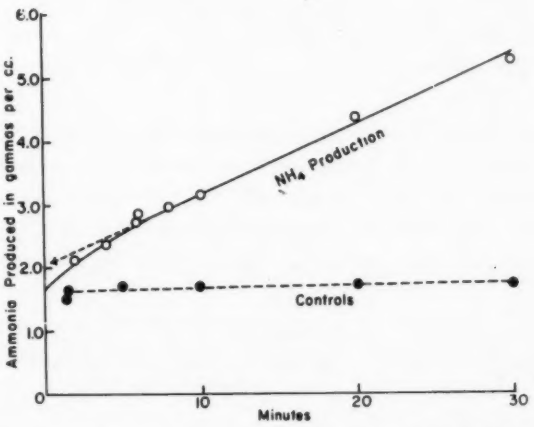

Fig. 3. The concentrations of ammonium achieved by alkalinization of plasma for increasing intervals of time is shown by the top line. The dotted line extended back to the origin indicates the concentrations of ammonia estimated to be present at time zero. The bottom line represents control values. The first plots are at 2 minutes.

produces additional color intensity, e.g., 0.007 u. of optical density in 25 minutes. The contribution of free ammonia from the alkaline hydrolysis of protein proceeds at a relatively slow rate,[5] only a small portion occurring in 3 minutes. Nesslerized eluent from the resin column after plasma increased 0.011 u. of optical density during the same period. Therefore, 0.007 u. of optical density may be attributed to maturation of the original ammonia–Nessler's complex and only 0.004 u. of optical density to further ammonia produced from the alkaline hydrolysis of protein and amino acids over 25 minutes. Since the samples are actually read at 5 minutes, this source of error is negligible.

Discussion

At pH 7.40 and room temperature, over 98 per cent of ammonia in solution exists as ammonium ion. In this method the ammonium is exchanged for excess potassium and sodium ions on cation exchange resins. At an elected time the ammonium is eluted from the resins, reacted with Nessler's solution, and its concentration measured colorimetrically.

Some ammonia is lost as shown by comparing the curve of directly Nesslerized standards with the curve of ammonia recovery from resins (Fig. 2). The distance between the 2 lines represents a portion of ammonium present on the resins but lost during elution. This portion diffuses as ammonia from the surface of alkaline drops below the column. The size of the drops and the time of exposure are constant enough to give a constant loss and a reproducible line. Using this line for interpretation, determinations of ammonia concentration are accurate to within plus or minus 0.1 μg per milliliter of ammonium nitrogen.

The chromatogram for the Nessler's–ammonia complex shows minimum transmittance at 378 mμ. It was elected to read above 400 mμ to avoid distortion from the glass cuvettes. Also, the resultant lower readings on the optical density scale are more widely spaced and easier to read. It is to be noted that inaccuracy in this wavelength setting will change the curve appreciably. A blank made of 0.1 N NaOH plus Nessler's reagent and ghatti gum begins to cloud after 20 minutes. Therefore, all standards and samples are read against water.

In the physiologic range this method is accurate and reproducible. In contrast to diffusion methods, ion exchange determinations can be accumulated after ammonium adsorption, and the rest of the procedure carried out on multiple samples at a convenient time. There is no rotator or mechanical equipment involved. There is no bedside procedure other than the drawing of the blood sample.

One other method[7] is based on the ammonia recovered from a protein-free filtrate. Although we have not set up this method for comparison, we feel that values may be falsely high because of the alkaline hydrolysis of unprecipitated amino acids during diffusion.

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A multihole needle for the aspiration of large quantities of bone marrow

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A multihole 13 gauge needle is described which has been found to be useful in obtaining large quantities of marrow from the posterior ilium of patients whose marrow yield is low when obtained with conventional needles.

The area in adult human subjects which yields the largest quantity of bone marrow is the posterior ilium. In most cases an ordinary 13 gauge needle and stylet suffice for removal of marrow from this area. However, not infrequently one finds the marrow yield in this region poor. This communication describes a needle with 16 holes in addition to the opening at the end of the barrel which is useful in obtaining more adequate quantities of marrow in such a situation.

Materials and methods

Description of the multihole needle. The instrument is a modified 13 gauge 3¼ inch trocar and cannula* (Fig. 1). The following modifications were made in the Machine Shop of the Sloan-Kettering Institute using standard catalog items. A needle point is fashioned as illustrated in Fig. 1. A row of 4 through-and-through perforations is made in the barrel of the needle, yielding 8 holes; each hole is $\frac{52}{71.000}$ of an inch (1.3 mm.). The holes are 5 mm. apart. The first hole is made 5 mm. from the heel of the beveled point. The second 2 rows of holes are drilled at right angles to the first and midway (2.5 mm.) between the first 2 rows of holes. The first 2 holes of the second set are made 2.5 mm. distal to those in the first 2 rows and are close to the proximal end of the beveled point. Thus, 16 holes in all are drilled.

The stylet is modified by placing on the shaft the head of the stylet used in the Bierman needle.†[1] This is comfortable for forceful thrusting, and the wheel-like shape provides leverage for turning. Since forceful pushing and turning are necessary to set the needle in

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*Becton, Dickinson and Co., Rutherford, N. J., Catalog No. 468 LRT.

†Becton, Dickinson and Co., Rutherford, N. J., Catalog No. H466 LNRH.

its proper location, the stylet set pin, which is extruded metal, is replaced with 2 metal pins firmly fixed to the shaft of the stylet. Suitable notches are cut in the hub of the needle to receive these. The 2 metal pins are strong enough to transmit the pressure of turning from the stylet to the barrel of the needle. It has not been our practice to siliconize needles.

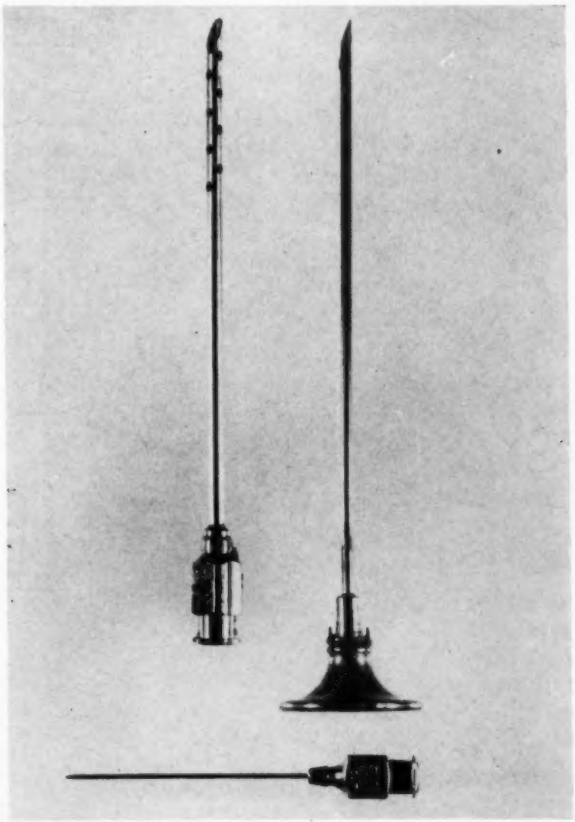

Fig. 1. The modified 3¼ inch needle. Eight of the 16 barrel perforations are visible. Note the 2 set pins in the Bierman-type head on the stylet. A 21 gauge 1½ inch needle is shown for comparison.

Clinical use. The technique of locating and aspirating from the region of the posterior iliac crest has been described and illustrated.[1, 2] We prefer that the patient lie prone while this area is aspirated. When the area is outlined, we customarily probe the site with a 21 gauge 1½ inch needle (Fig. 1). This gives us an idea of the depth at which bone will be struck and allows proper angulation of the needle. The needle and stylet are then inserted until the point is on the cortex of the bone. Since the distance from the point of the needle to the most proximal hole is about 3 cm., a silk tie on the barrel 3 cm. above the skin is a useful guide. We are not zealous about pushing the needle the full 3 cm. If marked resistance is encountered, we assume we have reached the cortex on the far side of the bone and push no further. Usually, the needle may be pushed a few more millimeters beyond the 3 cm. level and rotated to provide fresh aspiration sites. As an alternative to inserting the needle at right angles to the plane of the ilium, it may be inserted perpendicularly into the iliac crest parallel to any segment that has a straight plane for 3 cm.

Once the needle is in place the stylet is removed. Blood, rich with marrow particles and glistening with light reflected from the fat droplets, immediately wells up in the barrel and hub of the needle. A 30 or 50 ml. siliconized syringe is attached containing 2 ml. of

Table I. Comparative cell counts of marrow aspirates taken from various sites

Patient	*Site* aspirated*	*Volume*	*Nucleated cell count*	*Needle*
1. J. S.	Sternum No. 1	5 ml.	27,000	Rosenthal 16 gauge
(Normal	Sternum No. 2	5 ml.	42,100	Rosenthal 16 gauge
donor)	Anterior ilium No. 1	10 ml.	36,050	Rosenthal 16 gauge
W.B.C. 5,000†	Anterior ilium No. 2	10 ml.	19,500	Rosenthal 16 gauge
	Anterior ilium No. 3	10 ml.	21,950	Rosenthal 16 gauge
	Anterior ilium No. 4	10 ml.	5,700	Rosenthal 16 gauge
	Posterior ilium No. 1a	25 to 35 ml.	26,400	Multihole 13 gauge
	Posterior ilium No. 1b	25 to 35 ml.	15,500	Multihole 13 gauge
	Posterior ilium No. 1c	25 to 35 ml.	17,400	Multihole 13 gauge
	Posterior ilium No. 1d	25 to 35 ml.	15,800	Multihole 13 gauge
	Posterior ilium No. 2a	25 to 35 ml.	17,500	Multihole 13 gauge
	Posterior ilium No. 2b	25 to 35 ml.	15,950	Multihole 13 gauge
	Posterior ilium No. 2c	25 to 35 ml.	13,650	Multihole 13 gauge
	Posterior ilium No. 3a	25 to 35 ml.	21,950	Multihole 13 gauge
Total volume 605 ml.				
Nucleated cell count 16,300				
Total nucleated cell count 9.9×10^9				
Admixture of peripheral leukocytes 2.8×10^9				
Adjusted marrow yield 7.1×10^9				
2. A. P.	Sternum No. 1	5 ml.	56,000	Rosenthal 16 gauge
(Hodgkin's	Anterior ilium No. 1	15 ml.	87,150	Rosenthal 16 gauge
disease)	Posterior ilium No. 1	30 ml.	105,650	Multihole 13 gauge
W.B.C. 22,400	Posterior ilium No. 2	30 ml.	75,500	Multihole 13 gauge
Total volume 340 ml.				
Nucleated cell count 62,360				
Total nucleated cell count 21.2×10^9				
Admixture of peripheral leukocytes 5.4×10^9				
Adjusted marrow yield 15.8×10^9				
3. C. B.	Sternum No. 1	10 ml.	32,900	Rosenthal 16 gauge
(Normal	Sternum No. 2	10 ml.	32,650	Rosenthal 16 gauge
donor)	Right anterior ilium No. 1	15 ml.	12,350	13 gauge, no additional perforations
W.B.C. 6,100	Right anterior ilium No. 2	15 ml.	22,400	13 gauge, no additional perforations
	Left anterior‡ ilium No. 1	15 ml.	15,600	Multihole 13 gauge
	Left anterior ilium No. 2	15 ml.	11,800	Multihole 13 gauge
	Right posterior ilium No. 1	3 ml.	18,900	13 gauge, no additional perforations
	Left posterior ilium No. 1	20 ml.	32,150	Multihole 13 gauge
Total volume 410 ml.				
Nucleated cell count 14,250				
Total nucleated cell count 5.9×10^9				
Admixture of peripheral leukocytes 1.3×10^9				
Adjusted marrow yield 4.6×10^9				

*Letters after numbers refer to successive syringefuls from the same site.

†Peripheral leukocyte count.

‡The multihole needle was used on the anterior ilium by entering the ilium from above in the same plane as the bone.

heparin (crystalline heparin free of preservative, 30 mg. per cent in Gey's solution). Fig. 2 shows the multihole needle in place and bone marrow being aspirated. Aspiration need not be forceful and 30 ml. or more are obtained.

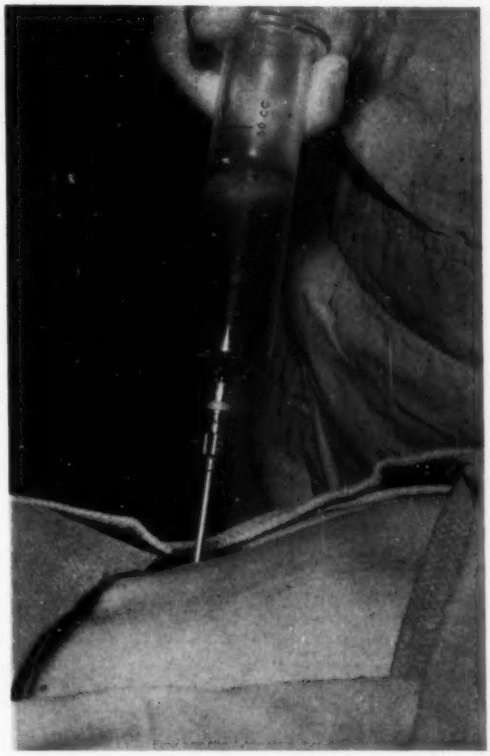

Fig. 2. The multihole needle in use. Note the marrow spicules suspended in the syringe contents.

Results

Table I illustrates comparative cell counts of marrow taken from various locations during multiple bone marrow aspirations. Results obtained with Patients J. S. and A. P. illustrate that aspirates with cell counts which compare favorably with those obtained from other areas may be achieved in larger volume with the multihole needle. Several syringefuls may be obtained but the nucleated cell counts of each successive syringeful may fall. Results obtained with Patient C. B. illustrate the particular usefulness of this needle. Bone marrow was obtained from this donor with difficulty. Marrow was aspirated from the right side of the anterior and posterior ilium with an ordinary 13 gauge needle, from the left side of the anterior and posterior ilium with the multihole needle. There was no advantage to the use of the multihole needle in the anterior ilium. In the right posterior ilium, repeated aspirations with the 13 gauge needle at various angles and depths yielded only 3 to 5 ml. of material. Using the multihole needle on the left, 4 syringefuls of 20 to 25 ml. each were obtained.

Kurnick[3, 4] has used a 13 gauge needle with 2 through-the-barrel perforations at the distal end for bone marrow aspiration; he has, however, abandoned its use because he is of the opinion that the perforations may weaken the needle. The multihole needle with 16 perforations, used with hand pressure, has given us no difficulty caused by weakness. The needle is never placed or advanced without the stylet. Force is transmitted from the head to the shaft of the stylet, the perforated barrel acting as a sheath for the stylet. On the other hand, we have found the 16 gauge Bierman needle, which is so satisfactory for diagnostic bone marrow aspirations, to be too fragile for the vigorous manipulations of the aspiration of large quantities of bone marrow from the ilium. In conclusion, it should be emphasized that one should not expect to obtain aspirates with higher cell counts with the use of this needle, but, in the patient from whom marrow is obtained with difficulty, the use of the multihole needle may be helpful.

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An improved colorimetric enzymatic assay of ceruloplasmin

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An improved colorimetric enzymatic assay of ceruloplasmin is described in detail. The conditions of the test provide an optimal combination of technical simplicity with precision and reliability of results. The values obtained compare favorably with those obtained by independent methods. Possible application of ceruloplasmin determination as a clinical screening test is discussed.

Ceruloplasmin, the blue, copper-containing alpha$_2$ globulin of human plasma and serum, elevates in association with chronic and acute infections, carcinoma, leukemia, and liver disease, and rises progressively in the course of normal pregnancy.[1] Elevated ceruloplasmin levels have been related to the presence of active major psychosis,[2] a finding partially confirmed in one subsequent study[3] but refuted by more recent investigations.[4, 5] Low ceruloplasmin levels are encountered in newborn infants, in some instances of the nephrotic syndrome (if there is a significant loss of ceruloplasmin in the urine), and in hepatolenticular degeneration (HLD, Wilson's disease).[1]

Clinical observations, however, have been generally restricted to laboratories especially equipped to conduct meticulous chemical, manometric, spectrophotometric, or immunochemical assays of this metalloprotein enzyme. A simple colorimetric assay for ceruloplasmin, based on the enzymatic oxidation of p-phenylenediamine (PPD) was reported in 1956 as an early practical diagnostic test for hepatolenticular degeneration, in which a profound deficiency of this metalloprotein is a highly specific, uniform, and probably basic feature.[6] The test devised at that time, however, was not sufficiently precise to permit application to serial observations of the less marked alterations in ceruloplasmin activity encountered in diseases other than HLD. Several colorimetric techniques, using PPD or N,N′-dimethyl-PPD, which have since been described,[2, 3] appear to be subject to significant sources of error.[4, 5] An improved modification of

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the first colorimetric ceruloplasmin method,[6] suitable for clinical application to problems other than HLD, is presented, therefore, with observations on the sensitivity, precision, and reproducibility of the procedure.

Materials

P-phenylenediamine-dihydrochloride (Eastman Kodak) is dissolved in a minimum volume of hot distilled water, decolorized with charcoal, filtered while hot, and recrystallized from the water-clear filtrate. The white crystals of purified PPD-2HCl are dried and stored in a vacuum over calcium chloride. The dried crystals may be kept in a sealed vial for several weeks without significant deterioration. A 0.5 solution of the purified substrate is prepared in distilled water just prior to use in the test.

A 0.4 sodium acetate buffer, adjusted to pH 5.5, may be prepared in large volume and should be stored at 4° C.

A 0.5 per cent solution of sodium azide in distilled water may be stored indefinitely at room temperature.

Methods

The test is performed on serum, preferably free of hemolysis or turbidity. Traces of hemolysis or turbidity are automatically compensated by the use of an enzyme-inhibited control, but a fresh specimen should be drawn if the optical density of 1:100 dilution of the serum in 0.4 M acetate buffer exceeds 0.100. If necessary, the serum may be stored at 4° to 10° C. for several weeks, or frozen, without alteration of the ceruloplasmin activity.

Siliconized glassware is employed throughout, except for collection of the blood sample, in order to provide more accurate transfer of small volumes of serum, and to reduce denaturation of the diluted serum proteins on glass surfaces. Samples of serum (0.1 ml.) are transferred with an 0.1 ml. pipette, 1 ml. volumes of substrate or of sodium azide introduced with 10 ml. Mohr pipettes, and 8 ml. volumes of buffer added with a 25 ml. Mohr pipette (or burette) in order to facilitate rapid handling of a large number of specimens while minimizing pipette errors.

Serum (0.1 ml.) is blown into the bottom of each of 3 siliconed 18 by 150 mm. test tubes. One milliliter of 0.5 per cent sodium azide is then added to one tube to provide an enzyme-inhibited control. Eight milliliters of buffer is added to all the tubes, followed by 1 ml. of an 0.5 per cent solution of substrate.

The substrate is added at the last step as rapidly as possible consistent with accurate pipetting. The test tubes are shaken to insure thorough mixing, placed in a test-tube rack, and immersed in a large, circulating constant temperature water bath at 37° C., to a depth

Table I. Effect of serum albumin on "spontaneous" and enzymatic oxidation of PPD*

Albumin† *(mg./10 ml.)*	*Ceruloplasmin*	*Optical density*	
		No enzyme	*Net*‡
0	0.640	0.180	460
2	0.588		
5	0.585		
10	0.558	0.108	450
20	0.535		

*Traces of copper introduced into the reaction with reagents may be shown to be catalytic of PPD-oxidation. The added albumin reduces "spontaneous" oxidation by combining with, and thereby reducing the concentration of, free ionic copper.

†Concentration of albumin in complete reaction mixture.

‡Note that the "net" oxidation, referable to enzyme, remains unchanged by addition of albumin.

corresponding to the height of the contents of the test tubes. After a 1 hour incubation period, the rack is removed and 1 ml. of 0.5 per cent sodium azide is added to each tube, *excluding* the control tube, in the same sequence as the addition of the substrate at the start of the test. The tubes are shaken once more to insure thorough mixing and are placed in a refrigerator for 30 minutes at 4° to 10° C.

The cooled reaction mixture is decanted into cuvettes of 10 mm. path, and optical density measured at 530 mμ. Under the conditions outlined above, virtually all normal and most abnormal sera will fall within the optimal working range of a spectrophotometer (O.D. 0.200 to 0.700). Samples with unusually high readings may be diluted with the buffer, and the reading corrected accordingly, rather than repeating the test with a smaller amount of serum.

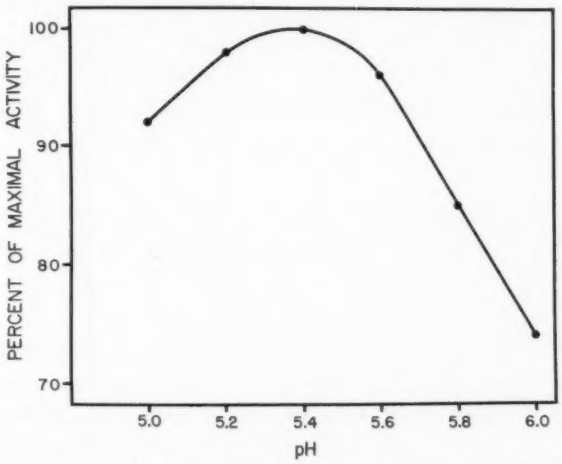

Fig. 1. pH-dependence of ceruloplasmin-catalyzed oxidation of PPD-HCl. Enzymatic activity is significantly higher at pH 5.5, employed in the colorimetric method, than at pH 6.0, employed in the manometric method. Identical curves were obtained with whole serum and with purified ceruloplasmin. The adsorption coefficient of the end product is unaffected by these variations in pH.

Results

Enzymatic oxidation of PPD by partially purified ceruloplasmin or by human serum is maximal at pH 5.4 to 5.5 (Fig. 1), which is employed in preference to the pH 6.0 buffer used in the Holmberg-Laurell[7] manometric procedure.[7] Evolution of the colored end product proceeds in a linear manner for at least 4 hours (Fig. 2), under the conditions described, to reach optical densities in excess of those encountered to date in any serum incubated for 1 hour. The rate of formation of the colored end product is directly proportional to the concentration of the enzyme over a wide range (Fig. 3).

A large, circulating water bath is required to minimize variability and delay in bringing the reaction mixture to proper temperature. Forced-draft hot air incubators or small water baths gave erratic results, while a 20 L. circulating bath regularly heated the contents of up to 48 test tubes to 37° C. within 3 minutes of immersion, from initial temperatures varying from 4° to 27° C.

The presence of a serum protein (albumin) other than ceruloplasmin markedly reduced the rate of spontaneous oxidation of PPD, but led to no reduction in the rate of enzymatic oxidation of PPD (Table I). Sodium azide

contributed no color to the reaction mixture, nor did it alter the rate of spontaneous oxidation of PPD in buffer or in buffer containing a plasma protein (albumin) other than ceruloplasmin (Table II). The addition of 1 ml. of 0.5 per cent sodium azide to 0.1 ml. of serum in the control tube leads to prompt and virtually *complete* inhibition of the enzymatic activity (Table II). At the end of the incubation period, however, the addition of 1 ml. of 0.5 per cent sodium azide to 0.1 ml. of serum in 9 ml. of buffered PPD does *not* result in immediate and complete cessation of enzymatic activity. If the control tubes and the sodium azide–inactivated enzyme assay tubes are allowed to stand at room temperature after removal from the incubating bath there is a progressive rise in optical density of the reaction mixture, more rapid in the "inactivated enzyme" tubes than in the corresponding control tubes, leading to an increasingly large over-estimate of enzymatic activity with delays in measurement.

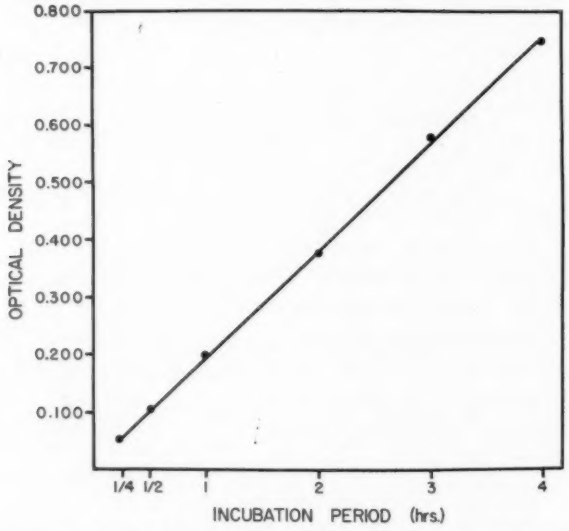

Fig. 2. Time course of ceruloplasmin-catalyzed oxidation of PPD-HCl. Note the absence of a detectable lag period, indicating that there is no significant competitive inhibition of the reaction by "endogenous substrates" added with the enzyme source. This curve obtained with whole serum in standard assay procedure.

Table II. Effect of sodium azide on "spontaneous" and enzymatic oxidation of PPD

*Sodium azide** *(mg./10 ml.)*		*Optical density*	
	Buffer	*Albumin*	*Ceruloplasmin*
0	0.120	0.070	3.400
1	0.120	0.070	0.131
5	0.124		0.092
10	0.118		0.072
20	0.122		0.061
50	0.119	0.070	0.050
100	0.120		0.056

*Concentration of sodium azide in complete reaction mixture.

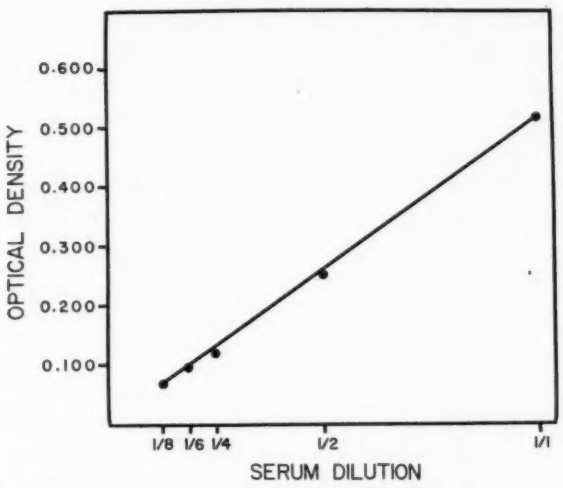

Fig. 3. Effect of enzyme concentration on reaction rate. Under the conditions selected for the clinical-laboratory test, PPD-oxidation retains a simple linear relationship to the amount of enzyme added. Variations in activity, therefore, are *directly* proportional to variations in enzyme concentration.

Placing the test tubes in a refrigerator immediately after the addition of sodium azide and standardizing the time interval between the end of the incubation period and spectrophotometry minimizes this source of error and eliminates rising optical density during spectrophotometry.

The attainment of reproducible test results requires rigid standardization of the *conduct* of the test procedure but only moderate precautions in handling serum samples. Serum may be stored at 4° C. for several weeks, or repeatedly frozen and thawed, without alteration in enzyme activity. However, if a clear serum becomes turbid or forms a precipitate during storage, it must be presumed that ceruloplasmin, as well as other proteins, may have been altered, and the specimen should be discarded. An almost total loss of PPD-oxidase activity, coincident with such changes in the physical state of the serum proteins, has been encountered in only a few sera out of a large number of stored samples tested.

When the test was performed in ordinary glassware, there were occasional large differences between samples, tested in triplicate, which could not be accounted for by errors in pipetting, incubation, enzyme inactivation, or spectrophotometry. These occasional unexplained variations in PPD-oxidase activity were eliminated by the use of siliconized glassware, as previously mentioned. With average manipulative skill, ceruloplasmin activity can be determined within a probable error of 2 per cent in a routine clinical application of the method.

In the absence of crystalline ceruloplasmin as a primary standard, a highly purified ceruloplasmin was calibrated by inactivation with sodium cyanide, and the loss of PPD-oxidase activity compared with the corresponding decrease in optical density of the ceruloplasmin preparation at 605 mμ in a Beckman DU

spectrophotometer (Table III). Using the Holmberg-Laurell factor[7] for conversion of optical density at 605 mμ to ceruloplasmin concentration (see footnote, Table III), the net optical density attained in the PPD-oxidase assay may be converted directly to milligrams per cent ceruloplasmin in whole serum by multiplying optical density by 87.5.

The routine test outlined in the method was carried out in 100 sera obtained at random from nonprofessional blood donors presumed to be free of disease by usual blood-bank criteria and, therefore, acceptable as blood donors. The mean PPD-oxidase activity of these sera was O.D. 0.369 ± 0.056 (1 S.D.), equivalent

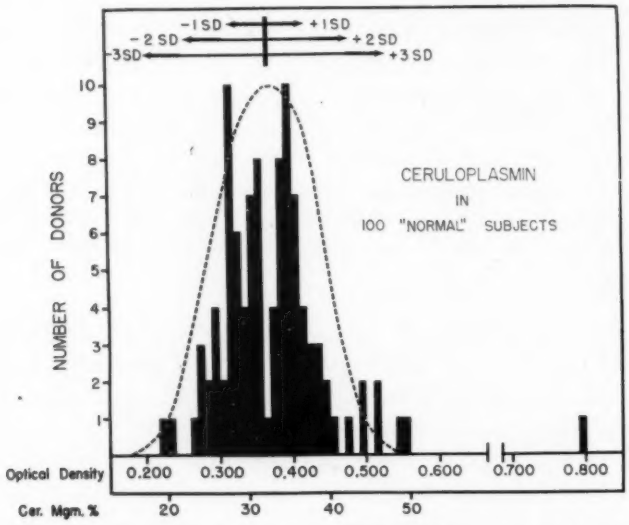

Fig. 4. Serum ceruloplasmin concentration (PPD-oxidase activity) in 100 presumed healthy blood donors. The bell-shaped curve represents the calculated Gaussian distribution of values, based on the arithmetic mean and standard deviation. The 3 sera falling outside the range of 3 S.D. above the mean nevertheless were included in this calculation, although they probably are *not* "normal, healthy" subjects.

Table III. Calibration of colorimetric PPD-oxidase activity as ceruloplasmin

Sample	*O.D. 605 mμ*	*PPD-oxidase activity*
Active enzyme	0.059	0.870
CN-inactivated enzyme	0.009	0.050

$$\frac{OD_{605} \times 4.585 \times 100}{0.32} = \text{mg. \% ceruloplasmin*}$$

$$\frac{\text{mg. \% ceruloplasmin}}{O.D._{530}\ m\mu} = \text{Factor for conversion of O.D. in PPD-oxidase test to mg. \% ceruloplasmin in serum in standard test.†}$$

*Derived from data of Holmberg and Laurell.[7] The constant 4.585 is the ratio between concentration of ceruloplasmin copper, expressed in micrograms per 100 ml. of a solution of pure enzyme, and the optical density, at 605 mμ, of the active enzyme in a 1 cm. path cuvette. The constant 0.32 is the concentration of copper in human ceruloplasmin, expressed in per cent.

†The mean value of this conversion factor, based on 6 determinations with 3 different preparations of purified ceruloplasmin, is 87.5. Thus, net optical density of PPD-oxidase activity of 0.1 ml. of serum in the standard test, multiplied by 87.5, expresses the result directly in the term "mg. ceruloplasmin per 100 ml. of serum."

to 32.3 ± 4.9 mg. per cent ceruloplasmin. This relatively small series of "normal" donors exhibited essentially a normal distribution of values but with 3 sera falling more than 3 S.D. above and no sera falling more than 2 S.D. below the mean of the entire group (Fig. 4.). A similar distribution with 4 sera out of 100 falling more than 3 S.D. above the mean had been obtained in an earlier series of randomly selected blood donors examined by a slightly less accurate modification of the test.

Discussion

The conditions selected for the present colorimetric assay method appear to provide a nearly optimal combination of simplicity of technique with a degree of precision and reproducibility suitable for a routine clinical and investigative procedure. The ceruloplasmin content of normal human serum determined by this method compares very well with the value of 34 ± 4 mg. per cent obtained by chemical analysis of "bound" serum copper[1] and 32 ± 4 mg. per cent determined by the immunochemical method on the same samples.[1] A normal value of 24 ± 10 mg. per cent obtained in another small series by the immunochemical method of assay[8] probably reflects the variability in batches of antiserum.

Other colorimetric enzymatic assays of ceruloplasmin have been described using either p-phenylenediamine,[3] N,N′-dimethyl-p-phenylenediamine,[2] or benzidine[9] in conjunction with higher concentrations of serum in the reaction mixture than employed here. Investigation of the first two methods, however, has clearly demonstrated that introduction of endogenous substrates, primarily ascorbic acid,[10] with the larger serum sample interferes with the assay by competition with oxidation of PPD or N,N′-dimethyl-PPD.[4, 5] Benzidine, on the other hand, is recognized to be too insensitive a substrate for ceruloplasmin, requiring a 12 to 24 hour incubation period for sufficient conversion to be readily measured spectrophotometrically.

A much wider application of this test must be carried out before the clinical significance of ceruloplasmin determinations can be properly assessed in conditions other than HLD. Premature claims of a significant relationship between ceruloplasmin levels and mental disease, subsequently invalidated, were primarily due to technical flaws in the methods employed for the assay of ceruloplasmin. The method described herein appears to be free of systematic error and to be absolutely specific for ceruloplasmin under the conditions outlined.

At the present time, ceruloplasmin determination is of diagnostic value only in hepatolenticular degeneration, in which there is, with rare exception, profound depression of the serum level. Low ceruloplasmin levels encountered in newborn infants (up to 3 months of age), in severe deficiency syndromes with malabsorption, and in some instances of the nephrotic syndrome with ceruloplasminuria (the only circumstance associated with urinary excretion of this protein-enzyme), are readily distinguished from hepatolenticular degeneration on clinical grounds.

The ceruloplasmin content of the serum of normal, healthy subjects appears to be remarkably constant, in a given individual, throughout the day and from

one day to the next.[11] Given a sufficiently reliable and sensitive method, therefore, variations in ceruloplasmin activity of serial samples should be susceptible to correlation with a clinical state under investigation. Thus, in a given individual, variation of more than 4 to 6 per cent (2 to 3 times probable error of the method) in the ceruloplasmin activity of successive sera may be presumed to be "real," i.e., a change in the status of the donor or an alteration introduced in the manipulation of the serum prior to testing.

The physiologic functions subserved by this enzyme remain completely unknown, although its ability to catalyze oxidation of histamine, adrenaline, noradrenaline, serotonin, ascorbic acid, and glutathione has been the basis for several speculative hypotheses regarding its function. Although ceruloplasmin copper has been shown to be exchangeable in the presence of ascorbic acid,[12] the enzyme does *not* appear to control gastrointestinal absorption of this trace element.

In view of the fact that ceruloplasmin elevation is encountered in association with neoplasms, chronic infection, recovery from myocardial infarction, etc., it might be well to study with more than average care the 3 per cent of apparently normal individuals who exhibit very high levels of this enzyme for no readily apparent reason.

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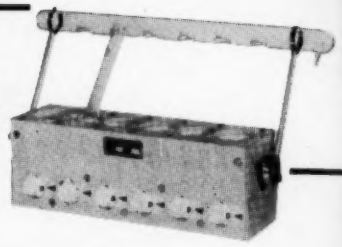

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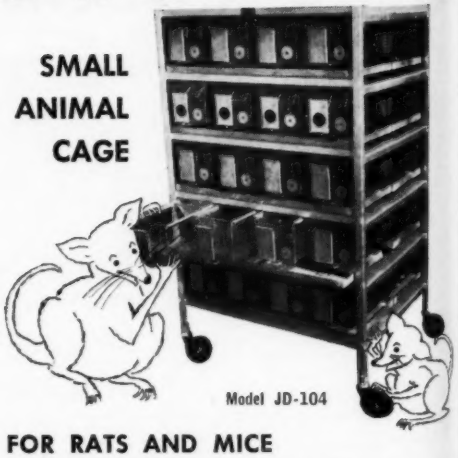

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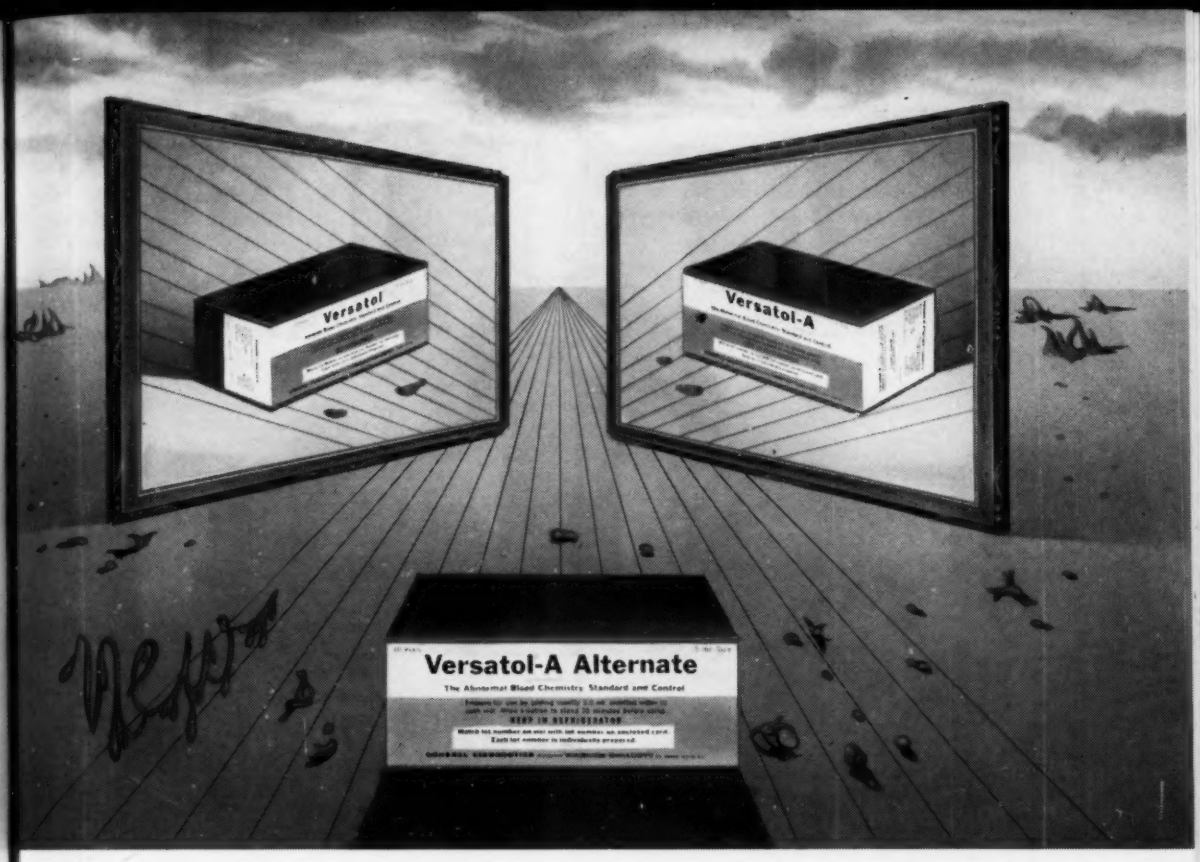

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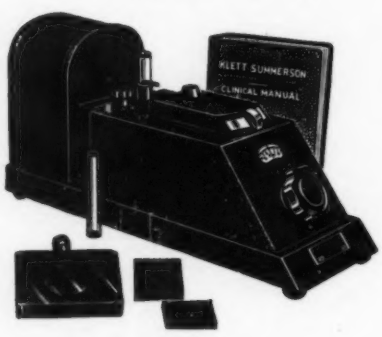

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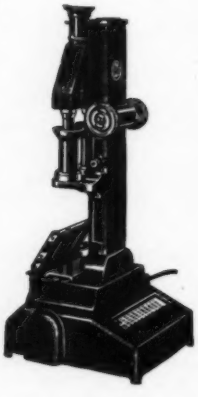

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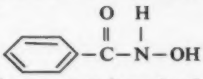
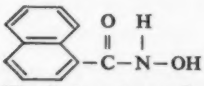


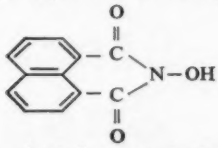
and it had held our attention. At this time our attention was also drawn to a new reagent for traces of calcium. It was called naphthalhydroxamic acid.

This, we mumbled to ourselves, figures—a homolog—



So we made it. The first customer found it worthless for the purpose. You, of course, realize why.

Our homolog is *1-Naphthohydroxamic Acid* and was so labeled. But who brought up the homolog jazz anyway? We did. The author who had proposed naphthalhydroxamic acid wasn't suffering from a speech defect or a typographical error. It was we who were suffering from the disease that causes jumping at conclusions. He meant the reaction product of hydroxylamine with naphth*al*ic anhydride, viz.



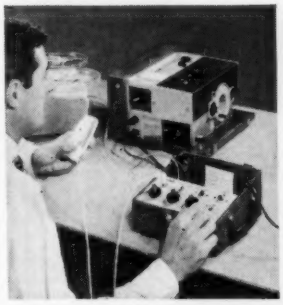
Abashed, we slunk back to the bench and made that. Another goof here would be most unbecoming. The author had said it was white. What color was ours? Yellow. Yellow, eh? Stall! Don't ship! Give any excuse, but don't ship! And we dug into the literature. Even dug up an old paper that speculated about what it called "xanthoisomers" of certain phthalic oximes otherwise structurally identical.

Our product *did* give the proper infrared absorption spectrum and melting point. It *did* give an excellent test for Ca^{++} when used in a procedure in *The Analyst* (Dec. '60), where the compound permits determination of calcium for 0.2 ml of serum as compared to the 2.0 ml required for accuracy by the standard oxalate method. This paper described the compound as yellow. A Japanese paper was also found that described it as yellow.

Confidence slowly returned. Under the name *N-Hydroxynaphthalimide* and the designation Eastman 8161, we are now releasing it and even offering procedural abstracts.

These are Eastman Organic Chemicals now. There are some 3900 Eastman Organic Chemicals. Substantial numbers of them are even stocked by our distributors across the country. List No. 42 is the latest. For a copy or for the procedural abstract on calcium write Distillation Products Industries, Rochester 3, N. Y. (Division of Eastman Kodak Company).

Multiply thyself



Should the thrill wear thin of skittering here and there over the map to show slides and speak your spiel, send out a box of slides, a *Kodak Cavalcade Projector*, a decent tape recorder, and a *Kodak Cavalcade Programmer* that ties them together in a presentation so alive that your own living presence will scarcely be missed. The time saved could be devoted to new efforts—raw material for slides yet unborn. In return for forgoing the pleasure of your company as entertainer, society gets more out of you as investigator.

The Kodak Cavalcade Programmer is used by a speaker to pre-package his presentation in solitude so as to maximize its cogency and minimize dependence on others for accuracy and effectiveness. Its panel bears three control knobs and a pushbutton. The microphone from the tape recorder is plugged into the programmer instead of the recorder itself. The magazine of the Cavalcade Projector is loaded with the slides. The speaker starts his speech. At the instant when he wants the first slide to come on, he presses the button. This records a 6.5-kc beep on the tape. Now he is seeing exactly what the audience will be seeing at this point in the discourse. Pertinent points about it may well occur to him that might not occur when speaking with his back to the picture and his gaze fixed on some vindictive-looking character in the fourth row. When he wants the next slide he presses the button again; without further human intercession the beep will change the slide at that instant every time. And so on for the allotted time. The "erase" feature of tape, a benign presence denied to those who speak in the flesh, is always comfortingly there during the recording session.

Why three knobs on the panel? We are glad you asked.

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The Kodak Cavalcade Programmer can also be used for presentations by audio-visual methods other than tape-and-slides. (It can cue a movie projector into a slide show, for example.) Conversely, it can be used with tape and slides for purposes unrelated to speechmaking. (It can cue a time-lapse camera, for example, by beeps spaced according to a table of random numbers.)

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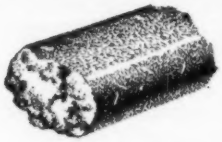

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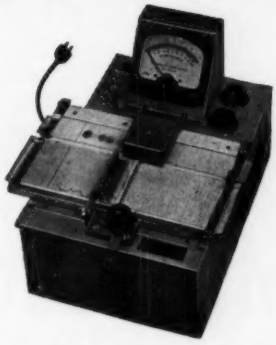

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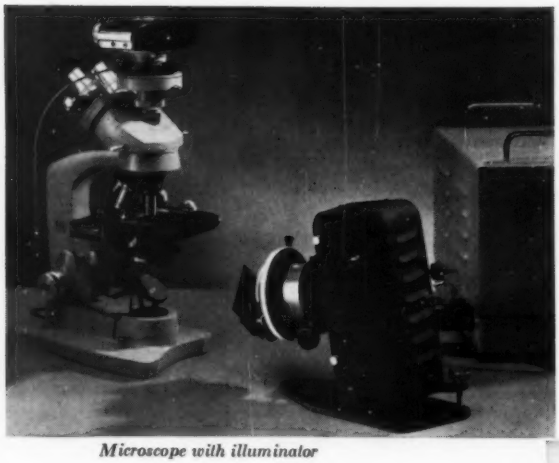
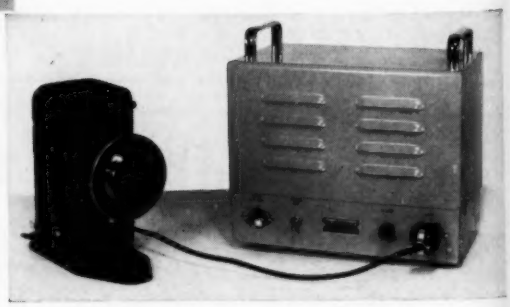


Microscope with illuminator



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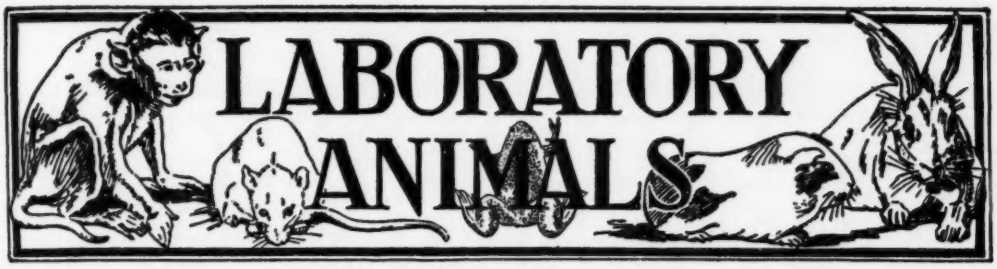

(See also pages 34, 35)

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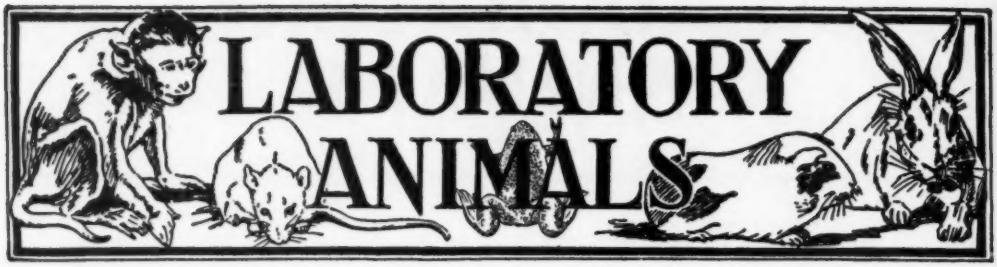

(See also pages 33, 35)

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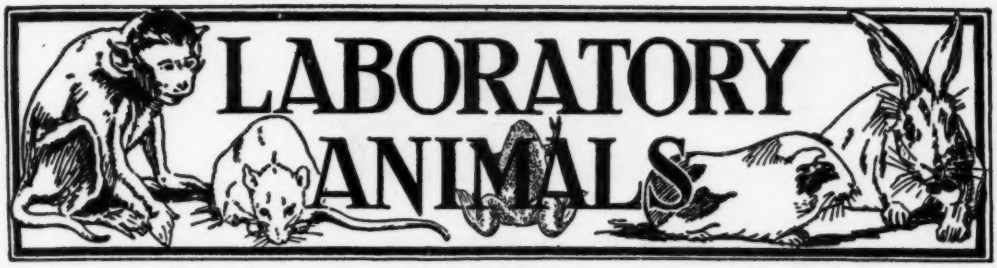

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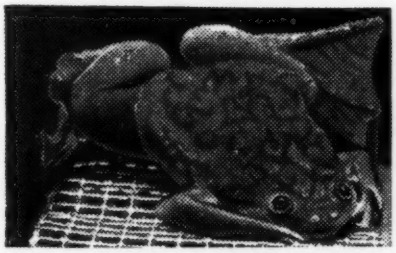

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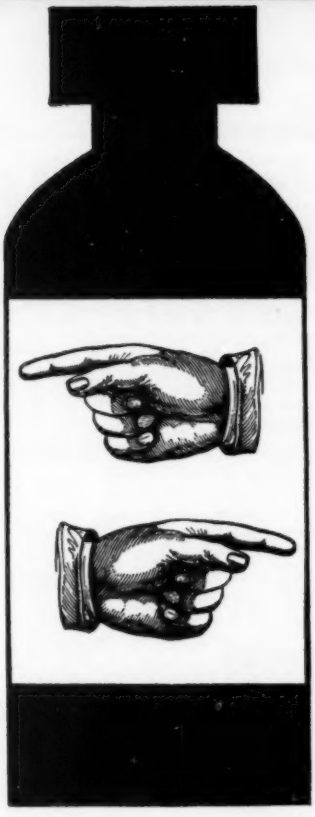

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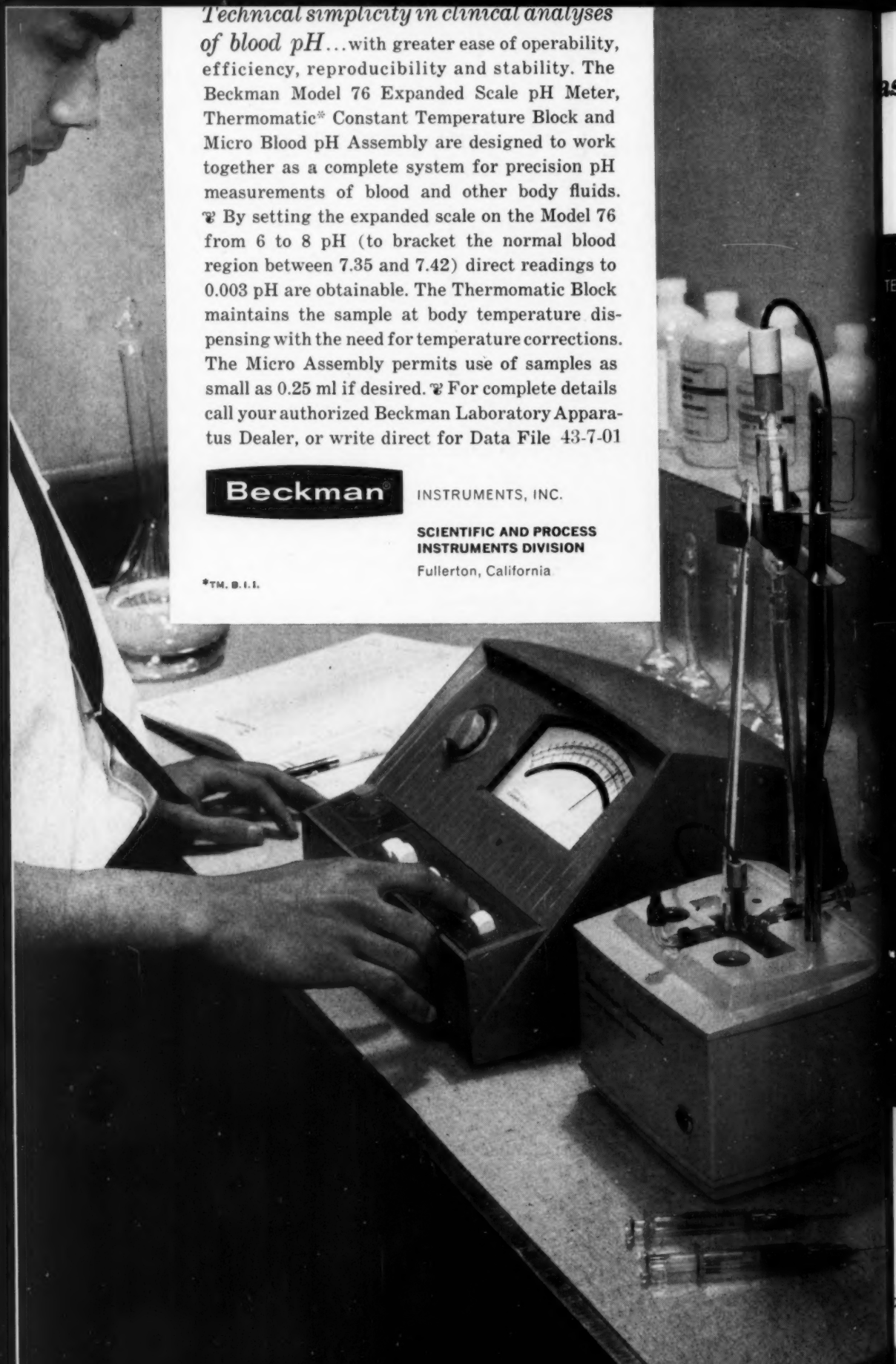

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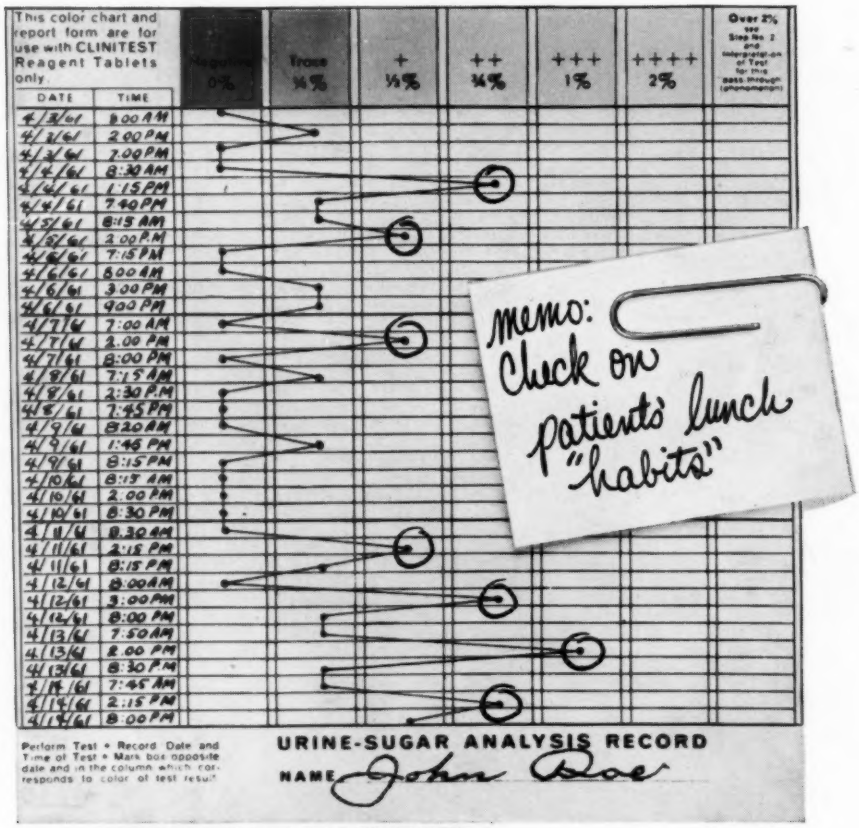
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